Analyses of hematopoiesis and lineage commitment from ES and iPS cells

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Meinen Eltern

So eine Arbeit wird eigentlich nie fertig,
man muss sie für fertig erklären,
wenn man nach Zeit und Umständen
das mögliche getan hat. *Zitat: Johann Wolfgang von Goethe*

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Abbreviations

AGM	aorta-gonad mesonephros
B cell	bursal- or bone marrow-derived cell
BAC	bacterial artificial chromosome
BCR	B cell receptor
BM	bone marrow
bp	basepair
CD	cluster of differentiation
cDNA	complementary DNA
CIAP	calf intestine alkaline phosphatase
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
D	diversity
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DMEM	Dulbecco’s modified eagle medium
DMSO	dimethyl sulfoxide
DN	double negative
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DP	double positive
dpc	days post coitum
DRFZ	Deutsches Rheumaforschungszentrum, Berlin
DTT	dithiothreitol
E. coli	Escherichia coli
EB	embroid body
EDTA	ethylenediaminetetraacetic acid
EF cell	embryonic fibroblast cell
ELISA	enzyme-linked immunosorbent assay
ES cell	embryonic stem cell
ETP	early thymis progenitor
FACS	fluorescence activated cell sorting
FCS	fetal calf serum

Flt-3	fms-like tyrosine kinase 3
Flt-3L	Flt-3 ligand
FSC	forward light scatter
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
H-chain	heavy chain
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HSC	hematopoietic stem cell
huCD25	human CD25
Ig	immunglobuline
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
iPS cell	induced pluripotent stem cell
ISP	immature single positive
J	joining
kb	kilobase
LB	lysogeny broth
L-chain	light chain
LIF	leukaemia inhibitory factor
lin	lineage marker
LMPP	lymphoid-primed multipotential progenitor
LSK cell	lin^- $Sca\text{-}1^+$ $ckit^+$ cell
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
MPIIB	Max Planck Institute for Infection Biology
MPP	mutlipotent progenitor
mRNA	messenger RNA
NK cell	natural killer cell
OD	optical density
PBS	phosphate buffered saline
PC	peritoneal cavity
PCR	polymerase chain reaction
pH	the negative logarithm (base 10) of the molar concentration of dissolved hydronium ions

pNPP	*para*-nitrophenylphosphate
preTα	pre- T cell receptor α chain
Rag	recombination activating gene
RANKL	receptor activator of NF-κB ligand
RBC	red blood cell
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase PCR
SCF	stem cell factor
sIgM / sIgD	surface IgM / surface IgD
SL chain	surrogate light chain
SP	single positive
SSC	side light scatter
T cell	Thymus-derived cell
TAE	tris-acetate-EDTA buffer
TCR	T cell receptor
TdT	thymidine desoxyribonucleotidyl transferase
TPO	thrombopoietin
TRAP	tartrate resistant acid phosphatase
U	unit
UV	ultraviolet
V	variable
YFP	yellow fluorescent protein
αMEM	alpha minimal essential medium
γ_C	common γ subunit of the interleukin IL-2, IL-4, IL-7, IL-9, and IL-15 receptor complexes

1 Abstract

An *in vitro* culture system has been developed that allows to differentiate embryonic stem (ES) cells into different types of hematopoietic, i.e. lymphoid, myeloid and erythroid, cell lineages. Cells were cultured on OP9 stromal cells for 5 days at which time they reached the mesodermal stage, characterised by Flk-1 expression. From day 5 to day 10, the addition of the cytokines SCF and Flt-3L resulted in generation of hematopoietic CD45-expressing progenitors that could be subcultivated under different conditions in order to induce differentiation of B and T lymphoid, NK, erythroid and myeloid cells.

The standardised *in vitro* culture system allowed a quantitative assessment of the capacities of different ES and iPS cell lines developing to erythroid, myeloid and lymphoid cell lineages.

It might have been expected that iPS cells generated from bone marrow-derived hematopoietic progenitor cells by ectopic expression of Sox-2, Oct-4 and Klf-4 would differentiate more efficiently than ES cells *in vitro* into hematopoietic cell lineages because of their epigenetic memory. Surprisingly, the efficiency to differentiate iPS cells to hematopoietic cells *in vitro* was found severely reduced compared to ES cells. In comparison to ES cells undifferentiated as well as differentiated stages of the iPS cell lines expressed elevated mRNA levels of the transcription factors Sox-2, Oct-4 and Klf-4 with which the iPS cells had been transduced.

These results indicate that overexpression of the transcription factors inhibits the development of Flk-1^{+} mesodermal to $CD45^{+}$ hematopoietic progenitors. The overexpression of Sox-2 appears to be inversely related to the hematogenic potential. These results suggest that iPS cell generation with the aim to develop hematopoietic cells should be controlled and selected for low levels of transduced Sox-2, Oct-4 and Kfl-4 expression.

In the second part of this thesis it was shown that long-term repopulating hematopoietic progenitors could be generated from ES cells using the established *in vitro* culture system. The development of progenitors was assayed by transplantation into sublethally irradiated $Rag2^{-/-}$ $\gamma_C^{-/-}$ recipient mice. Donor-derived B- and T-lymphoid and myeloid lineage cells found in bone marrow, thymus, spleen and peritoneum were characterised and quantified 4 months after transplantation.

Transplantation of ES cells differentiated for 9, 10 and 11 days, but not for shorter or longer periods of time, lead to long-term repopulation with donor-derived cells in bone marrow, spleen and peritoneum. Thus, transplantable hematopoietic progenitors could be found in the differentiation culture of ES cells only in a limited time window. This is the first report

showing long-term engraftment of immunodeficient mice with non-modified ES cell-derived hematopoietic progenitors.

Retroviral transduction of differentiating ES cells with HOXB4 allowed proliferative *in vitro* expansion of hematopoietic progenitors. Their long-term repopulating capacities were only 2- to 10- fold lower than those of normal bone marrow cells. In addition, it was found that even the thymus and the spleen were reconstituted with T cells, an *in vivo* differentiation capacity that had never been observed in transplantations with non-transduced differentiating ES cells. Interestingly, the *in vitro* differentiated ES cells could be stably transduced with HOXB4 as early as day 5, but not later than day 10 to yield reconstituting hematopoietic progenitors.

In the third part of this thesis reporter ES cell lines carrying bacterial artificial chromosomes (BACs) expressing YFP under the control of the preTα promoter and GFP under the control of the λ5 promotor have been generated. The analysis of *in vitro* differentiation into B and T lymphoid cells showed that the BAC λ5-GFP-transgenic ES cells became GFP^+ when they developed into preB cells but not when they developed into preT/T cells. By contrast, BAC preTα-YFP-transgenic ES cells became YFP^+ from the DN3 stage of T cell development on when they were differentiated on OP9-DL1 stromal cell in media containing IL-7 and Flt-3L, but stayed YFP^- when developed into $B220^+/CD19^+$ cells on OP9 stromal cells in media containing IL-7 and Flt-3L.

BAC preTα-YFP-transgenic mice were generated by pronuclear injection. They were crossed to existing λ5-huCD25 transgenic mice, and bone marrow and thymus were investigated for reporter gene-positive cells. YFP^+ $huCD25^-$ cells from the bone marrow were found in the common lymphoid progenitor (CLP) and proB cell stages. YFP^{high} $huCD25^-$ cells were found in the thymus resembling the DN3 T cell stage. Both populations were able to proliferate *in vitro* only under “T cell conditions”, but not under “B cell conditions”. On the other hand, YFP^- $huCD25^+$ from both organs had the potential to grow only under “B cell conditions” but not under “T cell conditions”. YFP^+ $huCD25^+$ cells found in the bone marrow had a proB cell phenotype ($B220^+$ $CD19^-$) and had a DP T cell phenotype ($CD4^+$ $CD8^+$) in the thymus. Both did not have the potential to proliferate *in vitro* under any condition tested. In summary, YFP marks T cell committed cells, while huCD25 marks B cell committed cells in these double transgenic reporter mice. For YFP^+ $huCD25^+$ cells neither B nor T cell potential could be determined.

2 Zusammenfassung

Die Kultivierung embryonaler Stammzellen (ES Zellen) und ihre Differenzierung zu verschiedenen Arten hämatopoietischer Zellen, u.a. lymphoiden, myeloiden und erythroiden, *in vitro* wurde optimiert und standardisiert, um eine zeitlich definierte, quantitativ auswertbare zelluläre Entwicklung zu verfolgen. Dazu wurden ES-Zellen auf OP9-Stromazellen für 5 Tage co-kultiviert, wobei sich mesodermale Flk-1^+ Zellen entwickelten. Von Tag 5 bis Tag 10 wurden die Zytokine SCF und Flt-3L zugegeben, so dass sich hämatopoietische CD45-exprimierende Zellen entwickelten. Diese hämatopoietischen Vorläuferzellen konnten danach unter unterschiedlichen Bedingungen weiterkultiviert werden, unter denen sich entweder B- oder T-lymphoide, NK, erythroide oder myeloide Zellen entwickelten.

Diese standardisierten *in vitro*-Zellkultursysteme ermöglichten eine quantitative Analyse der Kapazitäten verschiedener ES- und iPS-Zelllinien, die sich durch die Anzahl der gebildeten erythroiden, lymphoiden und myeloiden Zellen bestimmen ließen.

Es war erwartet worden, dass iPS-Zellen, die aus hämatopoietischen Knochenmarkzellen durch ektopische Expression von Sox-2, Oct-4 und Klf-4 generiert worden waren, aufgrund ihres epigenitischen Gedächtnisses *in vitro* effizienter in hämatopietische Zellen differenzieren als ES-Zellen. Erstaunlicherweise war die Effizienz der Differenzierung von iPS-Zellen im Vergleich zu ES-Zellen zu hämatopoietischen Zellen drastisch reduziert. Dabei wurde entdeckt, dass undifferenzierte als auch differenzierte iPS-Zellen höhere mRNA-Level der iPS-induzierenden Transkriptionsfaktoren Sox-2, Oct-4 und Klf-4 exprimierten als ES-Zellen. Diese Ergebnisse deuten darauf hin, dass Überexpression dieser Transkriptionsfaktoren die Entwicklung von mesodermalen Flk-1^+ zu hematopoietischen $CD45^+$ Vorläuferzellen inhibiert. Insbesondere die Überexpression von Sox-2 erscheint dabei wichtig zu sein: je höher die Sox-2-Expression, desto schwächer das hämatopoietische Potential der iPS-Zellen. Diese Resultate weisen darauf hin, dass die Generierung von iPS-Zellen zum Zweck der Entwicklung hämatopoietischer Zellen für niedrigere Sox-2-, Oct-4- und Klf-4-Expressionslevel kontrolliert und selektioniert werden sollte.

Im zweiten Teil dieser Arbeit konnte gezeigt werden, dass dieses standardisierte *in vitro*-Zellkultursystem dazu benutzt werden kann, langzeitig repopulierende hämatopoietische Vorläuferzellen aus ES-Zellen zu generieren. Die Entwicklung solcher hämatopoietischer Vorläuferzellen wurde durch Transplantation in sublethal-bestrahlte $Rag2^{-/-}$ $\gamma_C^{-/-}$-Rezipientenmäuse nachgewiesen. Die vom Donor abstammenden B- und T-lymphoiden und myeloiden Zellen wurden im Knochenmark, im Thymus, in der Milz und im Peritoneum

selbst 4 Monate nach Transplantation mittels ihrer spezifischen genetischen Markergene und ihrer Differenzierungsantigene charakterisiert und quantifiziert.

Transplantationen von ES-Zellen, die 9, 10 oder 11 Tage differenziert wurden, jedoch nicht kürzere oder längere Zeit, führten zu einer solchen Langzeit-Repopulation aus Donor-transplantierten Zellen im Knochenmark, in der Milz und im Peritoneum. Somit konnten transplantierbare hämatopoietische Vorläuferzellen in der Differenzierungskultur von ES-Zellen nur in einem beschränkten Zeitfenster gefunden werden. Dies ist die erste Studie, in der eine langzeitige Repopulation immunodefizienter Mäuse mit unmodifizierten aus ES-Zellen entwickelten hämatopoietischen Vorläuferzellen gelungen ist.

Retrovirale Transduktion von *in vitro* differenzierenden ES-Zellen mit dem chromatinremodellierenden HOXB4-Gen ermöglichte eine proliferative *in vitro*-Expansion der hämatopoietischen Vorläuferzellen. Deren Langzeit-Repopulationsvermögen in Knochenmarkstransplantations-ähnlichen Versuchen war nur 2- bis 10-fach geringer als die einer vergleichbaren Menge an normalen Knochenmarkszellen. Dabei wurde gefunden, dass sogar der Thymus und die Milz mit T-Zellen rekonstituiert waren, eine *in vivo*-Differenzierungskapazität, die in Transplantationen mit nicht-transduzierten differenzierenden ES-Zellen nicht beobachtet wurde. Interessanterweise konnten die *in vitro*-differenzierten ES-Zellen schon an Tag 5, aber nie später als Tag 10, stabil mit HOXB4 transduziert werden, um rekonstituierende hämatopoietische Vorläuferzellen zu erhalten.

Im dritten Teil dieser Arbeit wurden Reporter-ES-Zelllinien generiert, die bacterial artificial chromosomes (BACs) tragen, welche YFP unter Kontrolle des preTα-Promotors und GFP unter Kontrolle des λ5-Promotors exprimieren. Die Analyse der *in vitro*-Differenzierung zu B- und T-lymphoiden Zellen zeigte, dass BAC λ5-GFP-transgene ES-Zellen das GFP-Markergen exprimieren, wenn sie zu preB-Zellen wurden, aber nicht, wenn sie sich zu preT/T-Zellen entwickelten. Im Gegensatz dazu exprimierten BAC preTα-YFP-transgene ES-Zellen YFP vom DN3-Stadium der T-Zellentwicklung an, wenn sie auf OP9-DL1-Stromazellen in Medium mit IL-7 und Flt-3L differenziert wurden. Dagegen blieben sie YFP^-, wenn sie in $B220^+/CD19^+$ B-lymphoide Zellen auf OP9-Stomazellen in Medium mit IL-7 und Flt-3L differenziert wurden.

BAC preTα-YFP-transgene Mäuse wurden durch pronukleäre Injektion des Reportergenkonstruktes erzeugt. Diese Mäuse wurden dann mit vorhandenen λ5-huCD25-transgenen Mäusen gekreuzt und deren Knochenmark und Thymus auf Reportergen-positive Zellen untersucht. YFP^+ $huCD25^-$ Zellen wurden im Knochenmark im common lymphoid progenitor (CLP)- und proB-Zellstadium gefunden. YFP^{high} $huCD25^-$ Zellen wurden im

Thymus detektiert, die dem Phenotyp der DN3-T-Zellen entsprechen. Beide Zellpopulationen waren fähig, *in vitro* nur unter „T-Zell-Bedingungen“, aber nicht unter „B-Zell-Bedingungen“, zu proliferieren. Andererseits hatten YFP^- $huCD25^+$ Zellen aus beiden Organen das Potential unter „B-Zell-Bedingungen“, aber nicht unter „T-Zell-Bedingungen“ zu wachsen. Die YFP^+ $huCD25^+$ Zellen aus Knochenmark und Thymus konnten *in vitro* unter keiner der getesteteten B- und T-Zell-spezifischen Zellkultur-Bedingungen proliferieren. Dementsprechend markiert die einzelne Expression von YFP in den doppelt-transgenen Reportermäusen Vorläuferzellen auf dem Weg zur T-Zell-, aber nicht zur B-Zellentwicklung, während huCD25 umgekehrt Zellen auf dem Weg zur B-Zellentwicklung charakterisieren. YFP^+ $huCD25^+$ doppelt-exprimierende Zellen können weder in Richtung B- noch in Richtung T-Zell-Potential proliferieren.

3 Introduction

3.1 Embyronic stem (ES) and induced pluripotent stem (iPS) cells

3.1.1 Characteristics of mouse ES cells

ES cells, first isolated in 1981 from mice, derive from the inner cell mass of the blastocyst that develops 3.5 days of embryonic development and can still differentiate into tissues of all three germinal layers [1, 2]. Following microinjection into host blastocysts, donor ES cells contribute to all tissues of chimeric mice, including the germ line [3].

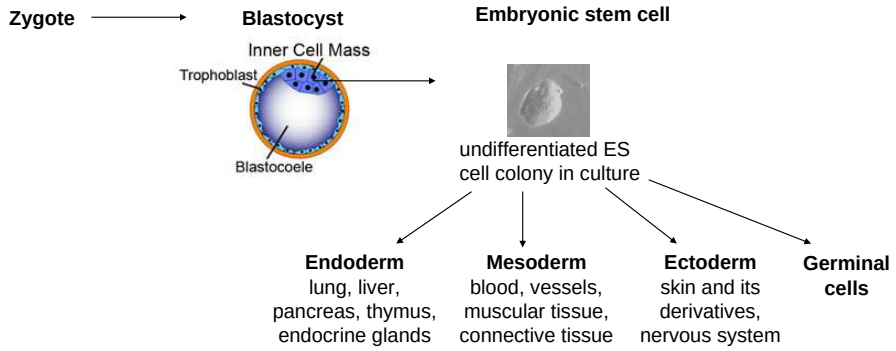

Figure 3-1: Embryonic stem cells derive from the inner cell mass of the blastocyst and can give rise to all three germ layers and germ cells.

This property, combined with the ability to easily manipulate the ES-cell genome via homologous recombination, provides the tools to generate new animal strains in which genes of interest are knocked out or altered.

Properties characterising ES cells are used as markers. ES cells have high alkaline phosphatase activity which is typically visualized by an enzyme-based reaction [4]. As all pluripotent cells they also have a high level of telomerase activity which can be measured by the telomeric repeat amplification assay [5]. A commonly used cell surface marker is SSEA-1 (stage-specific embryonic antigen) [6, 7]. The SSEA-1 epitope has been reported to play a role in cell-cell adhesion between blastomeres of the morula stage in mouse cells and is expressed on the surface of pre-implantation stage murine embryos (i.e. at the eight-cell stage), in inner cell mass cells and on the surface of teratocarcinoma stem cells, but not on their differentiated derivatives [7].

Several transcription factors, including Oct3/4 [8], Sox2 [9] and Nanog [10] function in the maintenance of pluripotency in both early embryos and ES cells. Genes like Stat-3 [11], E-Ras

[12], c-Myc [13] and Klf-4[14] have been shown to contribute to long-term maintenance of the ES cell phenotype and the rapid proliferation of ES cells in culture.

ES cells can be established and maintained in tissue culture in the state of their embryonic differentiation on feeder cell layers of mouse embryonic fibroblasts (MEFs) in the presence of the cytokine leukaemia inhibitory factor (LIF) [15, 16].

They hold the promise to be of therapeutic use for gene therapies of human genetic deficiencies and disorders. This is particularly promising for the hematopoietic cell system and its deficiencies, in which it has been possible to generate, from ES cells *in vitro*, transplantable progenitor cells with the capacity to populate erythroid, myeloid and lymphoid cell compartments in the transplanted host [17-22].

3.1.2 Generating ES cell-like cells from somatic cells - the iPS cells

Reprogramming of somatic cells can be induced by nuclear transfer into oocytes [23, 24] or by fusion with ES cells [25, 26]. Those experiments have implied that reprogramming factors can be identified in ES cells and be used to directly induce pluripotency in somatic cells. A rational approach led to the identification of four transcription factors, Oct-4, Sox-2, Klf-4 and c-Myc, enabling induction of reprogramming from adult fibroblasts [27].

To date, iPS cells have been generated from a variety of mouse as well as human differentiated tissue cells by the overexpression of the transcription factors Sox-2, Oct-4, Klf-4, in some cases with the added expression of c-Myc [27-33]. iPS cells exhibit many characteristics of ES cells, they are positive for alkaline phosphatase, they express the ES-cell specific surface marker SSEA-1, Nanog from the endogenous gene, can differentiate into all three germ layers and form teratomas upon injection into immunodeficient mice.

As ES cells and differentiated cells developed *in vitro* from ES cells are usually histoincompatible with the recipient a potential risk of transplantation rejection exists. Transplantation rejection can be overcome by using induced pluripotent stem (iPS) cells derived from the host who is to be transplanted. If iPS cells permit homologous recombination, as ES cells do, alteration of their genetic constitution by homologous recombination could be achieved by proper insertion of genes at experimentally selectable sites in the genome. Thus, it would be possible to repair the diseased person’s own cells.

If such iPS cells are functionally equivalent to blastocyst-derived ES cells they should allow the proper development of all cell lineages and their differentiated tissues of the body.

Although several reports indicate high similarities between ES and iPS cells, including indistinguishable global histone modification and DNA methylation patterns [29, 34], there is

increasing evidence for significant differences. The pattern of DNA methylation [35-37] as well as the expression of mRNAs and microRNAs [38-41] have been reported to be distinguishable between ES and iPS cells. Furthermore, the continued presence of an imprinted gene cluster, Dlk1-Dio3, might be mandatory for full developmental potential of iPS cells. [40]

The standard qualitative approach demonstrating reprogrammed somatic cells is to check germline chimera and teratoma formation from iPS cells [29, 31]. The question remains whether iPS cells are as efficient as ES cells to generate a whole mouse with normal life expectance, and to develop into somatic and progenitor cells at normal rates.

If iPS cells are to be used to develop hematopoietic cells for the reconstitution of a normal blood-forming system, as hematopoietic stem cells are capable in bone marrow transplantations and for the treatment of hematopoietic disorders they should be as efficient as ES cells in this development.

3.2 Hematopoiesis

3.2.1 Early hematopoiesis

Hematopoiesis during mouse development starts at 7.5 days post coitum (dpc) within the yolk sac originating from the mesoderm [42, 43]. The yolk sac predominantly supports primitive hematopoiesis whereas definitive hematopoietic cells mainly develop in the aorta-gonad-mesonephros (AGM) region and the fetal liver starting at 10 dpc [44]. Fetal liver hematopoietic stem cells (HSCs) eventually migrate to the bone marrow at around 16-17dpc [45] (Figure 3-2).

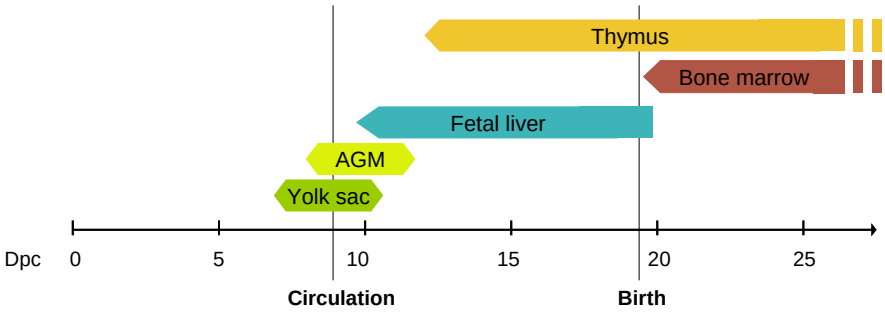

Figure 3-2: Stages of hematopoiesis in the mouse.

To follow hematopoiesis in embryonic development the markers Flk-1 and CD41, designating mesodermal differentiation, and CD45 and c-kit, identifying later hematopoietic development, can be used. Flk-1 is required for the generation of hemangioblasts, the stem cells for both

hematolymphopoietic and endothelial lineage cells [46, 47]. CD41 has been shown to be a marker for discrimination between endothelial and hematopoietic cells in the yolk sac of the mouse embryo [48, 49]. It has been used as a marker for the commitment to the hematopoietic lineage [50]. CD45 is a marker for all hematopoietic cells, including HSCs [45, 51-54], whereas c-kit is also expressed on undifferentiated ES cells.

HSCs are pluripotent stem cells that can give rise to all the different blood cell lineages. They are characterised by their self-renewing capacity, so that, upon division they retain their pluripotent stem cell property in at least one of the daughter cells. In appropriate environments they can differentiate into erythroid, megakaryocytic, myeloid and lymphoid cells. They have radioprotective and reconstitutive ability when they are transplanted into lethally irradiated hosts. Upon transplantation they home back to the bone marrow from where they can be re-isolated and transplanted into another recipient without loss of their stem cell properties [45, 53]. As HSCs divide, they form less potent progenitor cells including the common lymphoid progenitor (CLP), which gives rise to mature lymphoid effector cells including B, T, dendritic and natural killer (NK) cells [55]. The common myeloid progenitor (CMP) subset is capable of giving rise to mature myeloerythroid effector cells including monocytes/macrophages, granulocytes, megakaryocytes/platelets and erythrocytes [56].

To detect HSCs in mice transplantation assays have served as the most reliable method whereby cells where transplanted into irradiated hosts. The donor mice were analysed after a certain time for reconstitution of all the hematopoietic lineages [57-60]. To enrich and isolate HSCs from the adult bone marrow phenotypical analyses by flow cytometry based on cell surface marker expression have been performed [61-64]. HSCs have been highly enriched as Thy-1^{lo}Lin$^-$Sca-1^+c-kit^+ (LSK) or CD34$^-$Lin$^-$Sca-1^+c-kit^+ (LSK) cells [61, 65, 66]. The SLAM family members CD150 and CD48 were further identified to enhance HSC purity, hence 47% of the LSK CD150^+CD48^- population yield long-term multilineage reconstitution in irradiated mice [64, 67, 68]. During maturation HSCs loose the ability to self-renew while maintaining their full developmental potential. So called multipotent progenitors (MPPs) with more restricted self-renewing capacity have been identified as the LSK CD34^+Flt3^- population in the mouse bone marrow [69].

3.2.2 B and T lymphopoiesis

As the first stages in lineage commitment two distinct progenitor populations have been identified, the common myeloid progenitor (CMP) [56] and the common lymphoid progenitor (CLP) [55]. The common lymphoid progenitor (CLP) population is described as Lin$^-$IL-

7Rα^{+}ckit^{lo}Sca-1^{lo}Flk2^{+} can give rise to T cells, B cells, dendritic cells (DCs), and NK cells, but lacks myeloid and erythroid potential [55, 70].

CLPs further differentiate into B and T cells. While B cell development takes place in the bone marrow, T cells develop in the thymus.

In adult mouse hematopoiesis the cytokine interleukin 7 (IL-7) and its interaction with the high affinity receptor (IL-7R) is necessary for both B and T cell development [71-74]. The transcription factor EBF (early B cell factor) is the critical determinant of B cell fate decisions and collaborated with E2A [75-78]. Pax5 is suggested to be one of the target genes of EBF [77]. Pax5 in turn is only detected in B lymphocytes [79]. In Pax5$^{-/-}$ mice B cell development is arrested at an early proB/preB I-cell-like stage [80, 81].

The earliest type of B cell progenitor, the proB cell, is characterised by being B220^{+}Flt-3L^{+}CD19^{-}, NKI.1^{-} and CD4^{-} [82, 83]. EBF/E2A induces the expression of most B cell genes including mb-1 (Igα), B29 (Igβ), TdT, λ5 and VpreB1 & VpreB2, and the rearrangement of Ig heavy (H) gene locus leading to differentiation into preB I cells [76, 84]. PreB I cells are distinguishable by expression of B220, CD19 and Flt-3. In contrast to proB cells which have all their Ig loci in germline configuration the IgH-chain locus of preB I cells is D_HJ_H-rearranged at both alleles and germline IgL loci [85, 86]. Hence, Rag$^{-/-}$ mice have a block at the proB cell stage as rearrangement cannot occur. A block in B cell development at the preB I stage is found in mice that cannot express a preB-cell receptor or lack signalling molecules downstream of this receptor. The next developmental stage towards B cells is characterised by μH chain expression. Those preB II cells can phenotypically be distinguished by surface expression of CD25 and absence of c-kit and Flt-3. [87]. The preB II cells can be sub-divided into two subfractions: the large and the small preB II cells. The large preB II cells express the preB cell receptor (preBCR) consisting of the surrogate light chain (SLC) in association with the μH-chain on their surface [88, 89]. At this stage allelic exclusion of the second H-chain locus occurs while the rearrangement machinery is switched off [85]. Cells with a functional preBCR are positively selected by proliferative expansion, become small preB II cells, turn on Rag1 and Rag2 and induce V_LJ_L rearrangement of the light chains [85]. Small preB II cells that have undergone a productive L chain rearrangement can differentiate to immature B cells expressing the B cell receptor (BCR) consisting of a rearranged H and L chain (sIgM).

The majority of immature B cells home to secondary lymphoid organs and die while 5-10% differentiate here to mature B cells [83, 90-92]. Mature B cells are characterised by the upregulation of sIgD and are therefore double-positive for sIgM and sIgD.

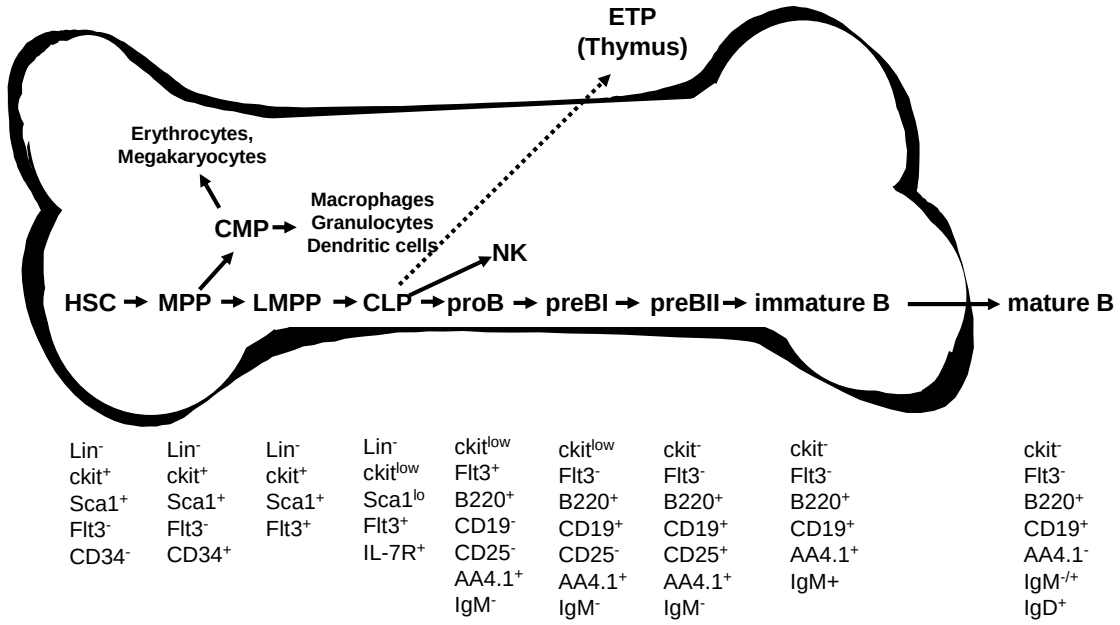

Figure 3-3: Hematopoiesis and B cell development in the bone marrow.

T-committed cells are evident in the blood [93]. Additionally, T-committed cells may be present in BM of adult mice [94]. The most immature intrathymic progenitors that have been identified are early thymic progenitors (ETPs) which possess B, NK and dendritic cell potential [95, 96]. Lymphoid progenitors enter the thymus at the cortico-medullary junction, then migrate while further differentiate to the outer cortex and end up in the medulla (Figure 3-4) [97-103]. The progression of cells can be traced by expression of CD4 and CD8 on the surface. First the cells do neither express one of them, they are called double negative (DN) thymocytes. Four stages can be distinguished based on surface expression of CD44 and CD25 – DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) [104]. At the DN3 stage, all cells are D-to-J rearranged at the T-cell receptor (TCR) β locus, which make them irreversible committed to the T lineage [105]. The TCRβ chain expressed from a productively rearranged *Tcrb* locus pairs with the invariant preTα chain to form a preT cell receptor (preTCR)[106-108]. At the same stage the *Tcrg* and the *Tcrd* loci rearrange. In case of productive rearrangement the TCRγδ complex, signalling stronger than the preTCR of αβ T cells, promotes γδ lineage choice[109, 110]. Impaired γδ signalling gives rise to αβT cell differentiation promoted by preTCR signalling to the immature single positive (ISP) stage, followed by upregulation of CD4 to become double positive (DP). At this stage the TCRα locus rearranges. TCRs that recognize self-peptide/self-MHC, hence they are positively selected, continue maturation becoming either CD4 or CD8 single-positive (SP) thymocytes [111-113].

During the whole process of T cell development Notch signalling plays an important role. It is essential for specifying the T cell fate and for promoting early T cell stages [114, 115] as well as directing CD4 versus CD8 T lineage commitment and αβ versus γδ T lineage decision [116-122].

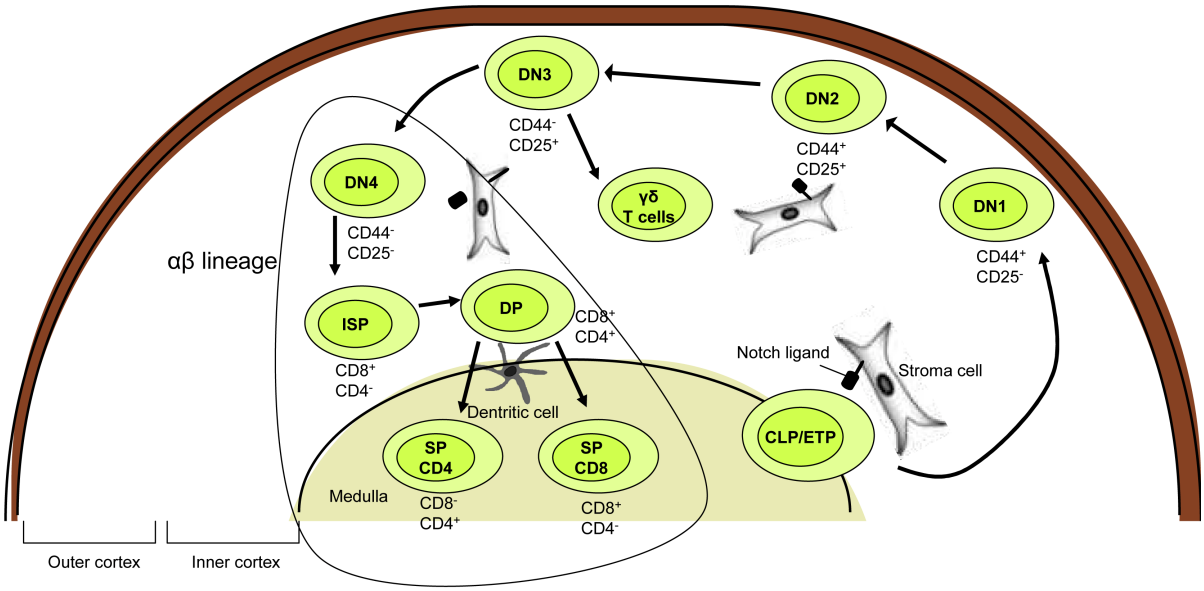

Figure 3-4: T cell development in the thymus (adapted from Anderson and Jenkinson Nature Reviews 2001).

3.2.3 The surrogate preB and preT cell receptor chains to follow B- and T-lymphopoiesis

During B cell development the heavy and light chain are V(D)J rearranged in a sequential manner [123]. In the proB cell stage D_H- to J_H-segments rearrange followed by V_H to D_HJ_H rearrangements [124]. Productive VDJ rearrangement gives rise to Ig_μ chains, expressed as a part of the preBCR. The preBCR is similar in structure to the BCR, consisting of two heavy Igμ and two surrogate light chains (SLCs) associated with the signalling subunits Igα and Igβ. The SLC is a heterodimer composed of two germline-encoded protein, VpreB1 or VpreB2 and λ5 [125, 126]. The genes encoding the proteins of the SLC are found on chromosome 16 in mice [127] and are coexpressed by the same cells [128].

Expression of λ5 has been monitored in mice that express huCD25 under the control of the λ5 promotor. The earliest progenitor in mice expressing transgenic huCD25 and mRNA encoding VpreB and λ5 is a $B220^+$ $CD19^-$ $CD27^+$ $ckit^+$ $Flt3^+$ $IL7R\alpha^+$ cell [129, 130] (Figure 3-5). When an Ig_μ is expressed from a productive V_H to D_HJ_H rearrangement it pairs with the SLC and forms the preBCR at the surface of preB II cells [131]. At this stage the transcription of the

SLC genes is turned off. The preBCR signals V_L to J_L arrangement, inducing further B cell development to IgM^+ immature B cells (Figure 3-5).

In a similar fashion, preT cells at the stage of DN3 express the preT cell receptor. The TCRβ chain pairs with the surrogate preTα protein. The gene encoding the preTα protein *Ptrca* is located on mouse chromosome 17 [132]. In contrast to the preBCR the surrogate chain of the preTCR consists only of one protein and has its own transmembrane portion. *Ptrca* is expressed at increasing levels by DN1, DN2, DN3 and DN4 thymocytes, whereby DN4 and DP cells rearrange their TCRα locus displacing preTα by TCRα forming the TCR [133] (Figure 3-5). High levels of preTα mRNA were observed in DP thymocytes, whereas preTα mRNA was

undetectable in SP thymocytes and peripheral (spleen and lymph node) T cells. Expression of TCRα precludes the formation of preTα/TCRβ dimers within the endoplasmic reticulum, leading to the displacement of preTCR from the cell surface ensuring that αβTCR expression on thymocytes undergoing selection is not compromised by the expression of preTCR.

To identify lymphoid precursors expressing preTα intracellularly a transgenic reporter mouse strain was used which expresses huCD25 under the preTα promoter [134]. Using the transgene preTα expressing cells were found in the bone marrow which were identified as being bipotent precursors of B and T cells [134, 135] (Figure 3-5).

The existence of a precursor coexpressing preTα and λ5 has been shown by RT-PCR. The question remains unclear whether this precursor can also be found using transgenes and which developmental potential it has [136].

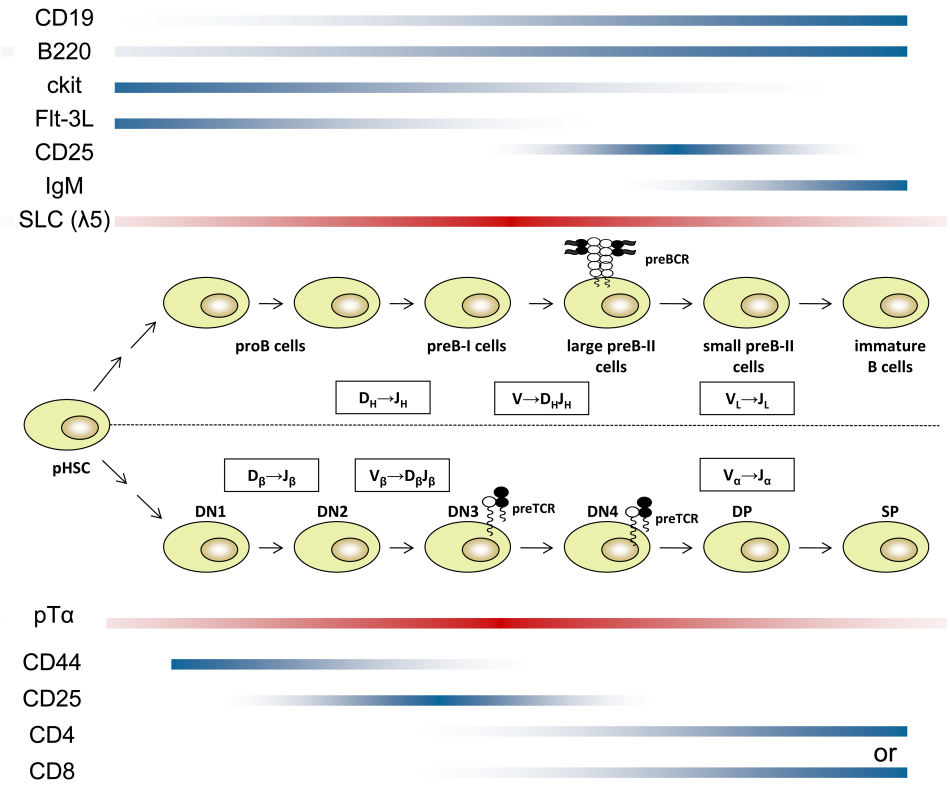

Figure 3-5: Expression of surface markers and preTα and λ5 in developing B and T lymphocytes.

3.3 Using ES and iPS cells to study hematopoiesis *in vitro* and for clinical application

3.3.1 In vitro generation of lymphoid, myeloid and erythroid cells from embryonic stem cells

Studying ES cells as they differentiate into lymphocytes, myeloid and erythroid cells *in vitro* is beneficial to gain insights into the cellular and molecular processes under various culture conditions, and facilitates manipulations of differentiating cells at various stages. Thus they are useful for studies of the genetics, cell biology, physiology and pathophysiology of the hematopoietic cell system.

For the *in vitro* development of myeloid and lymphoid cells from ES cells two protocols have been developed. One allows the formation of embryoid bodies [1, 137], the other the culture of ES cells on layers of stromal cell lines, notably the M-CSF-deficient OP9 cell line. [1, 138, 139].

Stromal cells partially support hematopoietic differentiation by cytokines that they produce. The absence of M-CSF favours lymphohematopoietic differentiation whereas addition of M-CSF results in myelopoiesis and decreases lymphopoiesis [138].

Cytokines shown to be essential for lymphoid development are stem cell factor (SCF), Flt-3 ligand (Flt-3L) and IL-7. SCF and Flt-3L appear to have a dual function in hematopoiesis in that they both have activity on stem cells and appear to act as critical early regulators of myelopoiesis/erythropoiesis and lymphopoiesis [140-142]. Flt-3L synergises with IL-7 to selectively stimulate lymphocyte development from uncommitted progenitors [143].

In vitro differentiation of ES cells on OP9 stromal cells yields mesoderm-like colonies after 5 days [138, 144, 145]. Addition of Flt-3L in the culture induced the development of hematopoietic blasts. Addition of IL-7 from day 8 of the culture on induces the cells to develop into B lineage committed cells which can be detected by expression of B220 [144, 145]. As mentioned above Notch signalling is essential for T cell development *in vivo*. Notch signals delivered by OP9 stromal cell ectopically expressing the Notch ligand Delta-like 1 (OP9-DL1) [146]. Following the same schedule for *in vitro* differentiation but using OP9-DL1 stromal cells induces T cell development [147].

Erythropoietin causes differentiation of progenitor cells into erythroid cells. *In vivo* it has been shown to act on two sites: first on an undifferentiated stem cell population in which it can induce differentiation, and on already differentiated basophilic erythroblasts increasing their rate of maturation [148-151]. In *in vitro* differentiation cultures it is therefore used to induce erythropoiesis from bone marrow as well as from ES cells [152, 153].

To induce myeloid cells addition of M-CSF is essential which is provided by ST2 stromal cells. Osteoclasts, representing one example of myeloid lineages, can be induced on ST2 cells starting with hematopoietic cells gained after 10 days differentiation of ES cells on OP9 stromal cells. An additional essential factor is receptor activator of nuclear factor κB ligand (RANKL) [154, 155] which is induced in ST2 cells upon addition of dexamethasone and 1α,25-dihydroxyvitamin D_3 [156-158]. Osteoclasts are detected by expression of tartrate-resistant acid phosphatase (TRAP) [157, 159]

3.3.2 In vitro generation of HSCs from ES cells

As ES cells can develop into all kinds of hematopoietic lineages *in vitro* it is expected that they transit through an HSC stage.

The development of HSCs and of differentiated hematopoietic progenitors from ES cells promises to be useful for clinical cell-based therapies [160-162]. However, the clinical applications of these HSC-based therapies are limited by the availability of histocompatible donors. Since it has now become possible to generate ES-like iPS cells from differentiated peripheral cells [27] histocompatible HSCs might in the future be generated from the

transplanted recipients own differentiated cells via iPS cells. Hence, there is practical need to further improve the conditions of *in vitro* ES cell differentiation to transplantable HSCs and more differentiated progenitors. Such efforts should include a quantitative assessment of the numbers of cells and their hematopoietic qualities.

To date, the most frequently used and broadly replicated method for the *in vitro* generation and expansion of hematopoietic progenitors from bone-marrow derived HSCs or from differentiating ES cells is their transduction with HOXB4 [18-22, 163-167]. HOXB4 allows long-term *in vitro* proliferative expansion of HSCs, apparently under the preservation of their functional capacities, possibly providing sufficient cell numbers for successful transplantations [17, 168].

However, genetic modifications always have the risk of side effects, e.g. leukaemia in the case of HOXB4 [169]. A few studies reported transplantation of non-HOXB4-transduced cells but none of them clearly show long-term engraftment of both, the lymphoid and myeloid compartment for 4 months [170-173].

However, efficient production of functional HSCs and more differentiated progenitors that can reconstitute all or parts of hematopoietic lineages *in vivo* remains difficult.

4 Thesis Objectives

The ability to generate hematopoietic cells from embryonic stem (ES) cells *in vitro* can facilitate genetic and molecular studies of this development.

Therefore, as the basis for all experiments performed during this thesis an *in vitro* culture system to differentiate lymphoid, myeloid and erythroid cells from ES cells should be developed.

Recent studies have shown that transcription-factor-based reprogramming of adult cells to an embryonic state yields induced pluripotent stem (iPS) cells that retain epigenetic memory [174]. Thus, iPS cells which have been generated from hematopoietic progenitor cells by ectopic expression of Sox-2, Oct-4 and Klf-4 are expected to differentiate more efficiently than ES cells into hematopoietic cell lineages because of their epigenetic memory. Therefore, in addition to overcoming transplantation tolerance, such hematopoietic cell-derived iPS cells, especially those derived from multipotent progenitors and stem cells of bone marrow, might be more efficient to generate hematopoietic cells.

The first aim of this study was to quantitatively monitor the development of hematopoietic lineages with time by determining the numbers of differentiated cells that develop at various times after initiation of ES- or iPS cell- differentiation *in vitro*.

The development of HSCs and of differentiated hematopoietic progenitors from ES cells promises to be useful both for clinical cell-based therapies as well as for studies of the genetics, cell biology, physiology and pathophysiology of the hematopoietic cell system. As all different kinds of hematopoietic cell lineages can be differentiated *in vitro* from ES cells it was expected that a stage of hematopoietic stem cells (HSCs) or hematopoietic transplantable progenitor must exist at some point in the culture. The differentiation is thought to occur in one wave. So, it might be that hematopoietic progenitors appear only in a short time window in the culture.

The second aim of this work was to find the right time point of appearance of transplantable hematopoietic progenitors in culture.

Therefore, cells from different days of the culture should be transplanted into immunodeficient $Rag2^{-/-}$ $\gamma_C^{-/-}$ mice to investigate their ability for lymphoid and myeloid reconstitution.

The third aim focussed on the decision point of lymphoid cells to become either B- or T-lineage committed and, in this decision, to determine whether early progenitors of the

two lymphoid lineages share expression of λ5 and preTα*, in vitro* by using the established culture system and *in vivo* by the generation of transgenic mice.

BAC clones for both genes were to constructed with YFP expressed under the control of the preTα promoter and GFP under the λ5 promotor. ES cells should then be transfected with the BAC clones to generate transgenic ES cell lines. I aimed at using those transgenic ES cell lines to develop B and T cells *in vitro* to study the lymphoid lineage commitment *in vitro*. In parallel, preTα-YFP/λ5-GFP BAC-transgenic mouse strains should be generated to study the appearance of developmental potential of cells expressing B- or T-cell specific genes *in vivo*.

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5 Materials and Methods

5.1 Material

5.1.1 Mice

All mice, but wild-type C57/

BL6 and BALB/c mice, were bred in the animal facility of the Max Planck Institute for Infection Biology, Berlin (MPIIB) under pathogen free conditions. Irradiated food and water were provided *ad libitum*.

Abbreviation	*Full strain designation*	*Notes and References*
C57/BL6	C57BL/6NCrl	Charles River
BALB/c	BALB/cAnNCrl	Charles River
Rag2$^{-/-}$ $\gamma_c^{-/-}$ C57/BL6	B6.129-Rag2^{tm1Faw}IL2-R$\gamma^{tm1.1Raj}$/J	
Rag2$^{-/-}$ $\gamma_c^{-/-}$ BALB/c	CByJ.129-Rag2^{tm1Faw}IL2-R$\gamma^{tm1.1Raj}$Foxn1^{nu}/J	
λ5-huCD25	B6.B6D2-Tg(Igll1-IL2RA)	[129]
preTα-YFP	C57BL/6-Tg(BAC-bMQ-353M7-preTα-nsYFP-pPGK-BSD)	developed during thesis
λ5-GFP	C57BL/6-Tg(BAC-bMQ-282I11-λ5-nsGFP-pPGK-BSD)	developed during thesis

5.1.2 Cell lines

5.1.2.1 Eukaryotic cell lines

Cell line	*Description*
J1 ES	wild-type ES cell line [175], derived from 129Sv mice (haplotype: H2K^b)
GK3 ES	D3 ES cell line [176], GFP knock-in the ROSA26 locus
Bruce4 ES	C56Bl/6-derived ES cell line, congenic for the Thy1.1 [177]; kind gift from Prof. Dr. Klaus Karjalainen, NTU Singapore
EF	primary cells, made in Berlin at the MPIIB from ED 14.5 embryos
OP9-J	MCS-F-deficient stromal cell line[138, 178, 179], kind gift of Prof.Dr. Zuniga-Pfluecker, University of Toronto

OP9-DL1	Delta-like 1 expressing OP9 stromal cell line [146, 180], kind gift of Prof. Dr. Zuniga- Pfluecker, University of Toronto
ST2	stromal cell line [181]
Plat-E	packaging cell line for retroviral viruses [182]
X63/IL-3	IL-3 producer cell line [183]
X63/IL-4	IL-4 producer cell line [183]
X63/IL-5	IL-5 producer cell line [183]
X63/IL-6	IL-6 producer cell line [183]
J558L/IL-7	IL-7 producer cell line [184]
CHO-SCF	SCF producer cell line [185], kind gift of Prof. Dr. T. Feyerabend, Universität Ulm
Sp2.0-Flt3-L	Flt-3L producer cell line[186], kind gift of Prof. Dr. P. Vieira, Institute Pasteur, Paris
J558-LIF	LIF producer cell line
M1	titration cell line for LIF[187]
5/7	IL-7 dependent, sromal cell independent cells line used for titration of IL-7, established in the lab

5.1.2.2 Bacterial cell lines

Cell line	*Description*
Top10	E.coli cell line[188], purchased from Invitrogen
Pir	E. coli cell line, deficient in production of a functional π protein needed for replication initiation (provided by R6K plasmids) [189]

5.1.3 Lab equipment

Device type	*Designation*	*Manufacturer*
Bi-Distillation Apparatus	Destamat Bi 18E	QCS
BioPhotometer		Eppendorf
Cell sorter	FACS Aria™	BD
Centrifuge	5810R and 5417R	Eppendorf
Clean bench	HERASafe® KS 9	Thermo Electron
CO_2 incubator	APT.LINE CB	Binder

Digital camera for microscope DFC 350 FX		Leica
Electric hook-up	MR3002	Heidolph
Flow cytometer	LSRII	BD
Freezers		Liebherr
Electrophoresis System	PerfectBlue™	Peqlab
Electroporation System	Gene Pulser Xcell™	Bio-Rad
Gamma irradiator	Gamma Cell 40	MDS Nordion
Gel analyzer	Gel Doc 2000	Bio-Rad
GFP/YFP filter set	510/21, 550/30, 525LP	Omega Opticals
Infrared lamp		Petra-electric
Laboratory ovens		Thermo Fisher Scientific
Liquid nitrogen tank	Arpege 170	Air Liquide
Microplate Reader	680 XR	Bio-Rad
Microscope (inverted)	DMI 400 B	Leica
Mixing device	Vortex-Genie® 2	Scientific Industries
pH meter	Seven easy	Mettler-Toledo
Pipettes and pipetting aid		Eppendorf
Power Supply	PowerPac Basic™	Bio-Rad
Refrigerators		Liebherr
Rotator	Roto-Shake	Scientific Industries
RT-PCR system	7900HT Fast RT PCR system	Applied Biosystems
Scales	Series XB	Precisa
Thermocycler	DNA Engine® PTC2000	Biozym Diagnostik
Thermomixer		Eppendorf

5.1.4 Disposables

Name	*Manufacturer*
Aspiration pipettes	Corning
Cell culture dishes	Nunc, ThermoElectron
Cell culture flasks	Corning, TPP
Cell strainer, 40µm	BD
Eppendorf tubes	Sarstedt
FACS tubes	Micronic
Falcon tubes	Sarstedt

Gene pulser cuvettes	Bio-Rad
Injection Needles	Braun
Maxisorp Plate for ELISA	Nunc, ThermoElectron
Multiple well plates (Costar®, TC-treated)	Corning
40µm mesh	BD
Omnifix syringe	Braun
PCR tubes	Sarstedt
Sterile pipettes (Costar® Stripette)	Corning
Sterile storage bottles	Corning
Surgical disposable scalpel	Roth
Vacuum driven bottle top filter 0.22µm	Millipore

5.1.5 Chemicals and Supplements

Name	*Manufacturer*
Acetone	Roth
Agarose(NEEO)	Roth
Bromophenol blue	Sigma-Aldrich
$CaCl_2$	Sigma-Aldrich
Compensation beads	BD
4′,6-Diamidino-2-phenylindol (DAPI)	Roth
Diethanolamine	Merck
Dimethyl Sulfoxide	Sigma-Aldrich
dNTP set (100mM)	Invitrogen
EDTA disodium salt 2 H2O	Roth
Ethanol	Roth
Ethidium bromide solution 0.5%	Roth
Fast red violet LB salt	Sigma-Aldrich
Formaldehyde	Roth
Gelatine	Roth
Glycerol	Sigma-Aldrich
HCl	Sigma-Aldrich
HEPES Solution	Sigma-Aldrich
KCl	Sigma-Aldrich
KH_2PO4	Sigma-Aldrich

2-Mercaptoethanol	Sigma-Aldrich
$MgCl_2$	Sigma-Aldrich
Na_2HPO4	Sigma-Aldrich
NaCl	Sigma-Aldrich
$NaHCO_3$	Sigma-Aldrich
NaOH	Sigma-Aldrich
Naphtol AS-BI phosphate	Sigma-Aldrich
Naphtol AS-MX phosphate	Sigma-Aldrich
N,N-Dimethylformamide	Sigma-Aldrich
oligodT	Fermentas
Paraformaldehyde	Sigma-Aldrich
p-Nitrophenyl phosphate (pNPP)	Sigma-Aldrich
2-Propanol	Roth
Random Hexamers	Fermentas
Sodium acetate	Roth
Sodium tartrate	Roth
TRIS	Roth
Trypan blue	Sigma-Aldrich
Tween20	Sigma-Aldrich
Ultra Pure™ Phenol:Chloroform:Isoamyl Alcohol	Invitrogen
Ultra Pure™ Buffer-saturated Phenol	Invitrogen

If not stated otherwise, all chemicals were purchased in p.a. purity.

5.1.6 Buffers, Media and Additives

Name	*Manufacturer/Composition*
αMEM	αMEM Powder (GIBCO® Invitrogen), 1.8g/l $NaHCO_3$, 1%(v/v) Penicillin/ Streptomycin 100x
$1\alpha,25(OH)_2D_3$	Biomol
Alkaline phosphatase staining solution	0.1mg/ml Naphthol AS-BI phosphate in 1% (v/v) N,N-Dimethylformamide, mixed until dissolved, then dissolved in 0.1M Tris-HCl pH 8.5, storage in the dark at 4°C, just before usage 0.16mg/ml Fast rad violet LB salt was dissolved
Ampicillin	Sigma-Aldrich, predissolved in water

BD FACS Clean Solution	BD
BD FACS Rinse Solution	BD
Blasticidin	PAA, predissolved in water
Chloramphenicol	Roth, predissolved in Ethanol
Dexamethasone	Sigma-Aldrich, predissolved in Ethanol
DMEM	DMEM/GlutaMax™, GIBCO® Invitrogen
Erythropoietin EPO	R&D
ESM	DMEM, 15% FCS, 0.1mM NEAA, 1% LIF, 0.1mM β-Mercaptoethanol, 1mM Pyruvate, 1% (v/v) Penicillin/ Streptomycin 100x
Ethidium bromide	Sigma-Aldrich
FACS Buffer	2% FCS in PBS
F-ESM	GMEM, 10% KSR, 1% FCS, 2mM L-glutamine, 0.1mM β-Mercaptoethanol, 1mM Pyruvate, 1% (v/v) Penicillin/ Streptomycin 100x
Gel loading buffer (6x)	30% Glycerol, 0.25% Bromophenol blue
Fetal calf serum (FCS)	Sigma, GIBCO® Invitrogen, JRH Bioscience (titrated for different cell lines)
Flt-3L recombinant	PeproTech
Gelatine solution	0.1% Gelatine in water, autoclaved
L-Glutamine 200mM	GIBCO® Invitrogen
Gentamycin	GIBCO® Invitrogen
GMEM	GMEM Powder (GIBCO® Invitrogen), 2.75g/l $NaHCO_3$, 1% (v/v) Penicillin/ Streptomycin 100x
Hygromycin B	Roche
IL-2 recombinant	PeproTech
IL-15 recombinant	Biochrom, predissolved in PBS
IMDM	IMDM Powder (GIBCO® Invitrogen), 3.02g/l $NaHCO_3$, 1%(v/v) Penicillin/ Streptomycin 100x, 50µM β-Mercaptoethanol, 1%(v/v) MEM non-essential amino acid (100x)
Kanamycin Sulphate	GIBCO® Invitrogen, predissolved in water
Knockout serum (KSR)	Life Technologies
Lipofectamine	Invitrogen

Lipofectamin 2000	Invitrogen
Lysogeny broth (LB) Media	1% (w/v) Bacto-Trypton, 0.5% (w/v) yeast extract, 85.5 mM NaCl, pH 7.5
Lysis Buffer for genomic DNA preparation	50mM Tris-HCl pH 8.0, 100mM EDTA, 100mM NaCl, 1% (m/v) SDS
Non-essential amino acids (NEAA) 10mM	GIBCO® Invitrogen
OptiMEM	GIBCO® Invitrogen
Puromycin	Calbiochem, predissolved in water
PBS (1x)	137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2
PBS (1x) for cell culture	Biochrom Cat.-No. L 1825
PBS-T	0.05% Tween20 in PBS
Penicillin/Streptomycin 100x	GIBCO® Invitrogen
Primatone RL	Quest
Proteinase K-(Tail-) buffer	50 mM TRIS (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% SDS
RBC Lysis Buffer	0.01M $KHCO_3$, 0.155M NH_4Cl, 0.1mM EDTA, pH 7.5
SCF recombinant	PeproTech
Sodium Pyruvate 100mM	GIBCO® Invitrogen
TAE (1x)	40 mM TRIS (pH 8.0), 1 mM EDTA
Tetracyclin	Sigma-Aldrich, predissolved in Ethanol
Thrombopoietin TPO	PeproTech, predissolved in PBS
TRAP staining solution	59.3M Sodium tartrate, 165.7M Sodium actetate, 0.5mg/ml Naphthol AS-MX phosphate, pH 5.0, storage in the dark at 4°C, just before usage 0.5mg/ml Fast rad violet LB salt was dissolved
Trypsin 2.5%	GIBCO® Invitrogen
0.05% Trypsin-EDTA	0.05% Trypsin (GIBCO® Invitrogen), 500µM EDTA, in PBS
0.1% Trypsin-EDTA	0.1% Trypsin (GIBCO® Invitrogen), 500µM EDTA, in PBS
0.2% Trypsin-EDTA	0.2% Trypsin (GIBCO® Invitrogen), 1mM EDTA, in PBS
0.25% Trypsin-EDTA	0.25% Trypsin (GIBCO® Invitrogen), 500µM EDTA, in PBS

5.1.7 Antibodies and Avidin Conjugates

Cognate Antigen	*Clone*	*Label*	*Notes*
Anti-human CD25	BC96	Pacific blue®	BioLegend
Anti-mouse B220	RA3-6B2	PeCy7, PE,	ebioscience
		Pacific blue®	ebioscience
Anti-mouse CD3	145-2C11	PerCP, FITC	ebioscience
Anti-mouse CD4	RM4.5	PE-Cy7, PerCPCy5.5	ebioscience
Anti-mouse CD5	53-7.3	Bio	ebioscience
Anti-mouse CD8α	53-6.7	APC, FITC	ebioscience
Anti-mouse CD11b (Mac-1)	M1/70	FITC,	DRFZ
		Pacific blue®	MPIIB
		APC	ebioscience
Anti-mouse CD11c	N418	Bio	DRFZ
Anti-mouse CD19	1D3	APC, PerCPCy5.5	ebioscience
Anti-mouse CD21	eBio8D9	bio	ebioscience
Anti-mouse CD23	B3B4	bio	ebioscience
Anti-mouse CD25	PC61.5	APC, PerCP-Cy5.5	ebioscience
Anti-mouse CD34	RAM34	bio	ebioscience
Anti-mouse CD40	FGK45	unlabelled	DRFZ
Anti-mouse CD41	eBioMWReg30	FITC	ebioscience
Anti-mouse/human CD44	IM7	Alexa450	ebioscience
Anti-mouse CD45	30-F11	FITC	ebioscience
Anti-mouse CD71	RI7 217.1.4	FITC	ebioscience
Anti-mouse CD93	AA4.1	Cy5	ebioscience
Anti-mouse c-kit (CD117)	ACK2	Cy5	ebioscience
Anti-mouse Flk-1	Avas12a1	PE, APC	ebioscience
Anti-mouse Flt-3 (CD135)	A2F10	PE, Bio	ebioscience
Anti-mouse Gr-1	RB6-8C5	APC	DRFZ
		Pacific blue®	DRFZ
Anti-mouse $H2K^b$	AF6-88.5	PE	BD
Anti-mouse IgA	polyclonal	unlabeled	Southern Biotech
Anti-mouse IgA	polyclonal	alkaline phoshatase	Southern Biotech
Anti-mouse IgD	11-26	Cy5, Pacific blue®	ebioscience
Anti-mouse IgG	polyclonal	unlabeled	Southern Biotech

Anti-mouse IgG	polyclonal	alkaline phoshatase	Southern Biotech
Anti-mouse IgM (μH-chain specific)	M41	Cy5, Bio	DRFZ
Anti-mouse IgM	polyclonal	unlabeled	Southern Biotech
Anti-mouse IgM	polyclonal	alkaline phoshatase	Southern Biotech
Anti-mouse IL-7rα (CD127)	A7R34	PE, bio	ebioscience
Anti-mouse MHC class II (I-A/I-E)	TIB120	Cy5	MPIIB
Anti-mouse NK1.1	PK136	FITC	ebioscience
Anti-mouse NKG2A/C/E	20d5	PE	ebioscience
Anti-mouse Sca-1	D7	PE	ebioscience
Anti-mouse Ter119	Ter-119	FITC, PE	ebioscience
Streptavidin		APC	BD
Streptavidin		FITC	ebioscience
Streptavidin		PE	ebioscience
Streptavidin		PeCy7	BD
Streptavidin		Qdot®605	Molecular Probes

5.1.8 Oligonucleotides

Name	*Sequence*
GFP/YFP rev	5’- AGCGCATGCTCCAGACTGCC-3’
GAPDH 5’	5’-TCTCCATGGTGGTGAAGACA-3’
GAPDH 3’	5’-GCAGTGGCAAAGTGGAGATT-3’
Granzyme 5’	5’-GATGTGAACCCTAGGCCAGA-3’
Granzyme 3’	5’-GTGGTAAGCATGCTCTGTGG-3’
Hbb (b-hemoglobin) 5’	5'-CACAACCCCAGAAACAGACA-3'
Hbb (b-hemoglobin) 3’	5'-CTGACAGATGCTCTCTT GG-3'
Hbb-bh1 (ζ-hemoglobin) 5’	5'-GCTCAGGCCGAGCCCATTGG-3'
Hbb-bh1 (ζ-hemoglobin) 3’	5'-TAGCGGTACTTCTCAGTCAG-3'
Hprt 5’	5'-AGTTCTTTGCTGACCTGCTG-3'
Hprt 3’	5'-GCTTTGTATTTGGCTTTTCC-3'
Klf4 FW	5’-AGAGTTCCCATCTCAAGGCACA-3’
Klf4 REV	5’-TCGCATTTTTGGCACTGGA-3’
Klf4_end_FW	5’-AGGAAGAGGAAGCGATTCAGGT-3’
Klf4_end_REV	5’-CGACTCACCAAGCACCATCAT-3’
λ5 3’ sequ	5’- TGAAGCTCCAGGGCCCACAG-3’

λ5 5’ sequ	5’- GTGGTGGAAACTAGAGACAGC-3’
λ5_HR_5	5’-AGATCTACACTGCAAGTGAGGCTAGAGTTGA CTTTGGACTTGAGGGTCAATGGTGAGCAAGGG CGAGGAGCTGTTCACCGGGGTGGTGCCCATC-3’
λ5_HR_3	5’-GAACTTCACACTGCCTGGGGATAGTGCCCAG AGTCTGTCCTACTCTGAGCTTGGGCTTAAGATAC ATTGATGAGTTTGGACAAACCACAACTA-3’
Nanog_FW	5’-AGCAGAAGATGCGGACTGTGTT-3’
Nanog_REV	5’-GAGTTCTTGCATCTGCTGGAGG-3’
Oct4 FW	5’-GGAGAATTTGTTCCTGCAGTGC-3’
Oct4 REV	5’-AGAACCACACTCGGACCACATC-3’
Oct4_end_FW	5’-CATTCAAACTGAGGCACCAGC-3’
Oct4_end_REV	5’-AATTTAACCCCAAAGCTCCAGG-3’
Perforin 5’	5’-GATGTGAACCCCTGGCCAGA-3’
Perforin 3’	5’-GTGGTAAGCATGCTCTGTGG-3’
preTα 3’sequ	5’-AGGCAAGAGGGACATACCTG-3’
preTα 5’sequ	5’-CTGGGGTACAGCTGGGTCTG-3’
preTα_HR_5	5’-CTAGCAGAGCCTCCCCCAACAGGTAGCTTCTG GCTGCAACTGGGTCATGGTGAGCAAGGGCGAGG AGCTGTTCACCGGGGTGGTGCCCATC-3’
preTα_HR_3	5’- CCCAGAAGCAGCAGCCATGTCCTAGCCATGG CCCACTCGTGGAGAAGGGGCTTAAGATACATTG ATGAGTTTGGACAAACCACAACTAGA-3’
Sox2_FW	5’-CGAGTGGAAATTTTGTCGGAG-3’
Sox2_REV	5’-CCTTCTTCATGAGCGTCTTGGT-3’
Sox2_end_FW	5’-CAAAAACCGTGATGCCGACT-3’
Sox2_end_REV	5’-AGACTTTTGCGAACTCCCTGC-3’

5.1.9 Enzymes

Name	*Manufacturer*
All restriction enzymes	NEB
Calf Intestine Alkaline Phosphatase (CIAP)	Fermentas
Phusion®	Finnzymes
Proteinase K, recombinant (PCR Grade)	Roche

Super ScriptIII® Reverse Transcriptase	Invitrogen
RNaseOUT. Recombinant RNase Inhibitor	Invitrogen
T4 DNA Ligase	NEB
Taq DNA Polymerase (recombinant)	Fermentas

5.1.10 Kits

Name	*Manufacturer*
QIAGEN Endofree plasmid maxi kit	Qiagen
QIAGEN Large-Construct Kit	Qiagen
QIAprep spin miniprep kit	Qiagen
QIAquick gel extraction kit	Qiagen
QuantiTect SYBR Green PCR Kit	Qiagen
SV Total RNA Isolation System	Promega

5.1.11 Software

Application	*Name/Manufacturer*
FACS analysis	Flow Jo, Tree Star
FACS acquisition & analysis	FACSDiVa 6.1, BD
Scientific graphics	Prism3.0, GraphPad software
Vector construction	GeneConstructionKit™2.5, TextCo

5.1.12 Vectors

BAC clones

bMQ-353M7 (contains mouse Igll1 (λ5) locus)

bMQ-282I11 (contains mouse Ptcra (preTα) locus)

The BAC clones were purchased from Geneservice.

Constructs

All pR6K construct were kind gifts of Prof. Francis Stewart, TU Dresden. The vectors were modified by normal PCR and restriction enzyme digestion techniques.

The retroviral vector expressing HOXB4 was kindly provided by Dr. Klaus Karjalainen, Nanyang Technological University, Singapore.

5.2 Molecular methods

5.2.1 Growth and storage of bacteria

Bacteria were cultivated in LB medium or on LB agar plates containing the appropriate antibiotic for selection of plasmids (100µg/ml Ampicillin, 15µg/ml Kanamycin, 20µg Chlormaphenicol, 4µg/ml Tetracycline, 30µg/ml Blasticidin, 40µg/ml Hygromycin B).

E. coli strains were stored as glycerol stocks at -80°C. Therefore, 0.85ml of an overnight culture of bacteria was vigorously mixed with 0.15ml sterile glycerol and the mixture was frozen to -80°C. To recover bacteria from frozen stocks, an inoculation loop was used to scratch off some bacteria which were subsequently streaked out on a LB agar plate containing the appropriate selection antibiotic and incubated overnight at 37°C.

To cultivate bacteria a single colony from a LB-agar plate was used to inoculate a microcentrifuge tube with a hole in the lid containing 2ml LB medium with the appropriate antibiotics. Bacteria were cultured overnight at 37°C shaking in a thermomixer at 900rpm unless otherwise noted. For large scale plasmid DNA preparation 500ml LB with the appropriate antibiotics was used and bacteria cultivated overnight in an Erlenmeyer flask.

5.2.2 Preparation of electrocompetent bacteria for transformation

Bacteria were recovered from frozen glycerol stocks as described above. 2ml LB medium with the appropriate antibiotic were inoculated with a single colony and incubated overnight shaking at 900rpm at 37°C. The next day 10% Glycerol and electroporation cuvettes were chilled on ice for at least 30min. 1.4ml LB medium with the appropriate antibiotic were inoculated with 50µl of the overnight culture and cultured for 2h shaking at 900rpm at 37°C. They should reach OD_{600}=0.6. The bacteria suspension was centrifuged for 1min at 10500g at 1°C. The supernatant was discarded and the pellet resuspended in 1ml chilled 10% Glycerol. This step was repeated once. The cells were centrifuged again and resuspended in 30µl chilled 10% Glycerol and kept on ice. The electrocompetent bacteria were usually directly used. The procedure could be scaled up and 0.1ml aliquots could be shock-frozen on dry ice and kept at -80°C until use.

5.2.3 Electrotransformation of competent bacteria

30µl of the freshly prepared or frozen electrocompetent bacteria suspension were transferred into a pre-chilled 1mm electroporation cuvette. 0.5 to 1µl of DNA was added and the content

of the cuvette mixed by vortexing. The bacteria were pulsed in a Bio-Rad Gene Pulser equipped with a Bio-Rad Pulse Controller set to 1.8kV, 200Ω, 25µF. The bacteria were resuspended in 1ml LB medium without antibiotics and cultured for 1h at 37°C as described above. Afterwards they were plated on LB agar plates containing the appropriated antibiotics and incubated overnight at 37°C unless otherwise noted.

5.2.4 PCR

The selective amplification of DNA sequences was performed with sense and antisense primers which were designed using the GeneConstructionKit™ software. They were usually between 22-35 bases long with a G,C-content ranging between 40%-60%. To provide cDNA with specific restriction sites at both ends for cloning, restriction enzyme specific recognition sequences were added to the 5' end of the oligonucleotide, followed by three additional unspecific nucleotides for efficient enzyme digestion. For analytic PCR reactions in which proofreading activity is not necessary Taq polymerase was used as followed.

100ng DNA were amplified in 1xPCR buffer with KCl supplemented with 0.2mM dNTP-Mix, 1.5mM $MgCl_2$, 1µM of each primer and 5U Taq DNA-Polymerase in a total volume of 30µl. The reaction was set up in 0.2ml PCR tubes. After short spinning of the samples PCR was carried out in a DNA thermal cycler at the following conditions.

Cycle	Temperature	Time	Step
1.	95°C	2min	Initial denaturation
2.-32.	95°C	30s	Denaturation
	45-65°C	30s	Annealing
	72°C	1s per 60 bp+10s	Elongation,
33.	72°C	5min	Denaturation
34.	10°C	∞	Storage

The samples were stored at -20°C until use. 10µl aliquots of PCR- products were mixed with 2µl Gel loading buffer (6x) and analysed on an agarose gel.

To prepare DNA by PCR for cloning for which the quality in terms of mutations is required, PCR reaction was set up using the Phusion polymerase as followed. 100ng DNA were amplified in 1x Phusion HF Reaction Buffer, 200µM dNTPs, 1µM of each primer and 2U Phusion polymerase in a total volume of 50µl in 0.2ml PCR tubes. After short spinning of the samples PCR was carried out in a DNA thermal cycler at the following conditions.

Cycle	Temperature	Time	Step
1.	98°C	30s	Initial denaturation
2.-35.	98°C	12s	Denaturation
	45-65°C	30s	Annealing
	72°C	1min per 1kb+10s	Elongation,
36.	72°C	10min	Denaturation
37.	10°C	∞	Storage

5.2.5 Small scale (mini-prep) and large scale preparation (maxi-prep) of plasmid DNA

For all preparations, anionic ion exchange columns were purchased from Qiagen together with necessary buffers. Small-scale preparations (mini-prep) for analytical purposes were carried out starting with pelleting the cells from 2ml culture and plasmid DNA was eluted from miniprep columns with 30µl water. Large scale preparations (maxi-prep) were carried out starting with pelleting the cells from 500ml culture and endotoxin-free plasmid DNA was precipitated and redisolved in 200µl water. The protocols for mini-prep and maxi-prep were performed according to the manufacturer`s instructions.

5.2.6 Restriction enzyme digestion of plasmid DNA or PCR-products

To get first information on the quality of plasmids after preparation of DNA, restriction enzyme digestion was performed. Therefore, usually 0.5µg DNA were digested with 5 units restriction enzyme in the recommended buffer according to the manufacturer in 30µl volume for at least 2h at the recommended temperature. After incubation the digestion of plasmid DNA was usually checked by conventional agarose gel electrophoresis.

To prepare vector backbones and PCR products for cloning, 2µg DNA were digested with 20 units restriction enzyme in the recommended buffer according to the manufacturer in 200µl for at least 4h at the recommended temperature. Vector backbones that were to be used for cloning were dephosphorylated using CIAP according to the manufacturer’s protocol.

5.2.7 Gel electrophoresis

1.5% (m/v) Agarose was mixed with 1xTAE buffer and heated in a microwave oven until the agarose was dissolved completely. The dissolved agarose was cooled down in a 65°C water bath. Before usage ethidium bromide was added at a final concentration of 0.1µg/ml.

Constant field agarose gel electrophoresis was used to separate DNA fragment in size range of 100bp to 12 kb. Gels were placed into a running chamber, which was filled with a sufficient volume of 1xTAE to submerge the gel. After loading of the samples electrophoresis was performed for 45-60min at voltages between 70V and 100V. To visualize DNA agarose gels were exposed to UV light and photographed.
For cloning appropriate fragments were cut with a scalpel from the agarose gel and purified using Qiagen Gel Extraction Kit according to the manufacturer's protocol.

5.2.8 Gel extraction of DNA

DNA fragments (amplified or digested DNA) were extracted from agarose gels using Qiagen Qiaquick Gel Extraction Kit following the manufacturer's instructions.

5.2.9 Ligation

Ligations of target vector backbones that receive the insert were performed in a total volume of 20µl. Usually the insert was added to a final molar insert:backbone ratio of 1:1, 3:1 and 5:1 and were incubated at 14°C overnight with 1µl T4-DNA ligase in 1x T4 ligation buffer. For every backbone a ligation negative control without the insert was performed to check the ligation background. Following ligation reaction suspension was frozen for at least 20min before electrotransformation into competent bacteria.

5.2.10 BAC DNA purification

Large scale preparations (maxi-prep) of BAC DNA were carried out starting with pelleting the cells from 750ml culture. DNA was purified using Qiagen Large-Construct Kit according to the manufacturer's instruction. Purified DNA was redisolved in 100µl water.

5.2.11 Generation of the targeting construct

All cloning steps regarding the vector to target the BAC for modification were performed in pir bacteria, which are deficient in production of a functional π protein needed for replication initiation[189]. This is provided by the vector backbone of all R6K plasmids. Cloning was done by conventional methods based on restriction enzyme digest and ligation as described above.

5.2.12 Preparation of the targeting cassette

The targeting construct were linearized by restriction enzyme digest (enzyme should cut in the backbone of the targeting construct) overnight (20µg in 200µl digestion volume). The fragment was purified by Ethanol precipitation. Therefore, 20µl 3M NaAc and 600µl 96% Ethanol was added to the digested construct. The mixture was centrifuged at 15500g at 4°C for 20min. The pellet was washed with 500µl 70% Ethanol and centrifuged again for 10min. The pellet was air-dried and resuspended in 12µl water.

5.2.13 BAC modification by homologous recombination

Bacterial artificial chromosomes (BACs) contain large inserts which offer several advantages for functional genomics. Conventional cloning methods rely on the use of restriction enzymes which precludes engineering of such large molecules.

Zhang et al. developed a strategy to modify BACs by Red/ET recombination, a method which is based on homologous recombination *in vivo* in E.coli mediated by the *rec*E and *rec*T proteins (Zhang et al. 1998). A distinct locus can be modified by recombination with a PCR product containing homology arms of 50nt. The technique has been extended using the pBAD-αβγ plasmid in which *rec*E and *rec*T have been replaced by their functional counterparts of phage lambda *redα* and *redβ* (Muyers et al. 1999).

Stewart et al. (unpublished) have further improved the method to modify the BAC by using DNA oligonucleotides containing homologous regions of both, the targeted locus and the modification cassette of the targeting construct. Oligonucleotides consisting of at least 80nt with at least 40nt homologous to the targeting cassette and 40nt to the targeting site in the BAC were ordered in diluted to a concentration of 50µM.

First of all BAC clones (Chloramphenicol resistant) were recovered from frozen glycerol stocks and made electrocompetent as described above. 1µl (0.2-1µg/µl) of the Red/ET recombination protein expressing plasmid was added to 30µl of electrocompetent bacteria and the whole mixture transferred to the chilled electroporation cuvette. The bacteria were electroporated as described above, resuspended in 500µl LB and returned to the microcentrifuge tube. To allow the bacteria to recover the tubes were incubated at 30°C for one hour at 900rpm. The cells were afterwards plated on LB agar plates containing 20µg/ml Chloramphenicol and 5µg/ml Tetracycline and incubated overnight at 30°C.

To start overnight culture a colony was picked from the plate and 2ml LB medium with 20µg/ml Chloramphenicol and 5µg/ml Tetracycline was inoculated. The cultures were incubated at 30°C at 900rpm. The 1.4ml LB medium with 20µg/ml Chloramphenicol and

5µg/ml Tetracycline was inoculated with 50µl of the overnight culture and cultured for 2h shaking at 900rpm at 30°C. To induce the expression of the Red/ET Recombination proteins 20µl 10% L-arabinose was added to the tubes and incubated for 40min at 37°C shaking at 900rpm. The cells were prepared for electroporation as described above. 2µg of the purified cassette and 1.5µl (150pmol) of each DNA oligonucleotide was added to 30µl of electrocompetent bacteria and the mixture transferred to an electroporation cuvette. The bacteria were electroporated as described above, resuspended in 500µl LB and incubated at 37°C for one hour at 900rpm to allow homologous recombination to occur. The cells were plated on LB agar plates containing 20µg/ml Chloramphenicol and the appropriate antibiotic selecting for the insertion of the cassette and incubated overnight at 37°C.

5.2.14 Verification of successfully modified BACs

To find clones that are correctly recombined 10-20 modified and 2 unmodified control BAC colonies were picked and BAC DNA was isolated by mini-prep as described above. The whole DNA from a 2ml culture was digested with one frequently cutting restriction enzyme (either XhoI, NcoI, PvuI or PstI) overnight. At least 2 different digests were done per clone. The whole digested DNA was loaded on a 1.5% agarose gel and analysed for additional or missing bands in control to the unmodified BAC DNA.
The insertion region of modified BAC clones were sequenced by *Dr. Martin Meixner* (*SMB*, Rüdersdorf).

5.2.15 Preparation of genomic DNA

1-5x10^6 cells were lysed for at least 4h at 37°C by adding 1ml of Lysis Buffer and 10µl Proteinase K to the cell pellet. After that 1ml Ultra Pure™ Buffer-saturated Phenol was added and the mix for 30min on a rotator. The mix was centrifuged at 15500g for 3min and the upper phase transferred to a new tube. 1ml Ultra Pure™ Phenol:Chloroform:Isoamyl Alcohol was added, again mixed for 30min on a rotator and centrifuged at 15500g for 3min. The upper phase was transferred to a new tube, 1ml of 2-Propanol was added and centrifuges at 15500g for 3min. The supernatant was aspirated and the pellet was washed with 70% Ethanol. After centrifugation at 12000rpm for 3min, the supernatant was aspirated, the pellet was dried and resuspended in 20µl water.

5.2.16 Preparation of tail DNA and genotyping

Tail biopsies from mice were digested in 750µl tail buffer containing 30µl proteinase K (400µg). Incubation was carried out in a thermomixer at 400rpm, 55°C for at least 4h. After incubation 250µl 6M NaCl were added, mixed and centrifuged at 18500g for 10min. The supernatant was transferred into a fresh Eppendorf tube and DNA was precipitated by adding 600µl 2-propanol with subsequent centrifugation at 18500g or 10min. The supernatant was discarded and the pellet was washed with 70% ethanol. The final pellet was resuspended in 50µl water. The DNA was analysed by PCR reaction as described above.

5.2.17 Purification of RNA

RNA of $2x10^5$- $5x10^6$ cells was isolated using the SV Total RNA Isolation System following the manufacturer's instructions. Purified RNA samples were stored at -80°C.

5.2.18 RT-PCR

For qualitative mRNA expression a two-step protocol was used (1^{st} step: cDNA synthesis, 2^{nd} step: target gene amplification). For cDNA synthesis 0.1-1µg RNA diluted in 10.5µl water were converted into first strand cDNA. First 50pmol OligodT, 0.2µg Random Hexamers and 10nmol dNTPs were added and incubated for 5min at 65°C and placed on ice for 1min. 7µl of the cDNA Synthesis Mix (4µl 5x RT Buffer, 0.1µmol DTT, 40U RNase Out, 200U SuperScript III) were added and incubated at 25°C for 5min, 50°C for 1h, 70°C for 15min and stored at 10°C until freezing at -20°C. For target gene amplification a normal PCR was done using Taq DNA Polymerase as described above.

5.2.19 Quantitative RT-PCR

Expression levels of mRNAs were quantitatively measured by quantitative real- time RT-PCR using the QuantiTect SYBR Green PCR Kit in a 7900HT Fast Real-Time PCR system with the HPRT gene as reference. For each reaction (25µl final volume), 0.25µl RT mix and 10µl of RNA sample (50ng-100ng/reaction) were mixed with 0.5µl of primer pairs (20µM each), 12.5µl of SYBR Green mix and 1.75µl RNase-free H_2O. Each sample was assayed in triplicate for every run. Control RNA from wild-type cells or an indicated specific positive control was used to construct a standard curve for all inspected genes, proving specificity and reliability of the designed oligonucleotide pairs.

The following reaction conditions were used:

Cycle	Temperature	Time
1.	50°C	30 min
2.	95°C	15 min
3.-47.	95°C	20 sec
	60°C	40 sec
	72°C	40 sec
48.	95°C	15 sec
49.	60°C	15 sec
50.	95°C	15 sec

5.3 Cellular work

5.3.1 Freezing and thawing of cultured cells

Cells which were intended for storage were resuspended in 1ml freezing media and transferred into 1.5ml freezing vials. The vials were put into a -80°C refrigerator and kept for at least 1 day. For long-term storage, frozen stocks were transferred into liquid nitrogen.
Thawing of frozen cells was accomplished by incubating the vials in a 37°C water bath. The cell suspension was immediately transferred into appropriate culture medium, centrifuged at 290g for 5min and resuspended in fresh media. Cells were cultured under maintenance conditions for the appropriate cell line.

5.3.2 Determination of the cell number (counting)

An aliquot of cell suspension was diluted 1:2 or 1:10 (dependent on the cell number) with trypan blue solution to distinguish dead cells and living cells. Living cells were counted using a Neubauer counting chamber (0.1 mm depth). The number of cells per ml was calculated as following:

$$\textit{Number of cells per ml} = \textit{Number of cells counted per mm}^2 \times 10^4 \times \textit{dilution}$$

5.3.3 Maintenance of OP9 and OP9-DL1 stromal cells

The adherent stromal cell lines OP9 and OP9-DL1 were cultured on 145mm cell culture dishes with αMEM/20% FCS in a humidified incubator at 37°C, 5% CO_2 and passed every 2

to 3 days at around 90% confluency. Cells were washed twice with PBS and treated for 5min at 37°C with 0.1% Trypsin/EDTA per dish to detach the cells from the dish. The reaction was stopped by addition of 15ml αMEM/2% FCS followed by centrifugation at 290g for 5min and resuspension in fresh culture media. Stromal cells were counted and replated at $3.5x10^5$cell /145mm dish.

To prepare OP9 or OP9-DL1 cells for cocultivation of differentiating cells $2.3x10^4$ cells/cm^2 for 100% or $1.2x10^4$ cells/cm^2 for semi-confluency were seeded into culture flasks or dishes accordingly for usage on the same day. It was assumed that cells divide ones a day, hence numbers were calculated for preparation one, two or three days in advance of usage. Usually OP9 cells were used without irradiation, but if noted they were irradiated with 3000rad.

5.3.4 Maintenance of ST2 stromal cells

ST2 cells are usually cultivated on 145mm cell culture dishes in RPMI medium supplemented with 5% FCS and 50µM β-Mercaptoethanol. When reaching 100% confluency they were washed once with PBS, 3ml 0.05 %Trypsin/EDTA per dish was added and incubated for 2min at 37°C. The reaction was stopped by addition of 10ml media and usually $3.5x10^5$ cells per 145mm dish were seeded.

5.3.5 Preparation of embryonic fibroblasts (EF cells)

Embryos from embryonic day 14.5 were prepared and transferred to PBS in a petri dish to remove blood. The head and internal organs were removed, remaining parts were washed twice in PBS, transferred into 10ml PBS and cut into very small pieces. 10ml 0.2%Trypsin/EDTA was added and tissue fragments were incubated at 37°C for 10min. After vigorous pipetting, they were again incubated at 37°C for 5min. Following removal of tissue debris 20ml DMEM/10% FCS was added and the whole mixture was passed through a 40µm mesh to remove remaining debris. The cells were then centrifuged at 290g for 5min, the supernatant was aspirated the cells resuspended in DMEM/10% FCS. Per embryo one 100mm dish was prepared to cultivate cells in 10ml DMEM/10% FCS. To remove debris 4h after seeding the media was changed and the cells were further cultivated overnight. The next day cells were frozen in freezing vials in FCS/ 10%DMSO as cells from one embryo to one vial.

To prepare irradiated EF cell stocks cells from one embryo were thawn on a 145mm dish and cultivated in DMEM/10% FCS. When reaching 90% confluency cells were passed. Therefore cells were washed twice with PBS and incubated for 5min at 37°C with 0.05%

Trypsin/EDTA. The reaction was stopped by adding 15ml culture medium followed by centrifugation at 290g for 5min. EF cells were resuspended and plated on three 145mm dishes. When reaching again 90% confluency cells were passed to nine 145mm dishes as described before. Passing the nine dishes as described before included counting cells and seeding at least $2x10^6$ cells on a maximum of 27 dishes. In the final step the cells were harvested at 100% confluency as described before. After resuspending them in fresh media they were γ-irradiated with 3000rad, which prevents further cell division during co-culture with ES cells. Cells were frozen at cell numbers of $1.5x10^6$.

5.3.6 Production of growth factor conditioned hybridoma cell culture supernatants

The hybridoma cell lines were grown in IMDM/2% FCS/0.03% Primatone. When the pH indicator in the culture medium turned from red to orange (lower pH) and approximately cells started dying the cells where centrifuged at 290g for 5min, the supernatant was sterile filtered and frozen in aliquots.

To find the concentration of the supernatants for cell culture they were titrated using cytokine-sensitive cell lines and compared with the efficient concentration of control recombinant cytokines.

5.3.7 Maintenance of embryonic stem (ES) cells

ES cells were usually cultured on 60mm dishes on an irradiated EF cell layer. Therefore, 60mm dishes were treated with 5ml gelatine solution for at least one hour. Irradiated EF cells were thawn as described above. The next day EF cell media was aspirated and ES cells were thawn. Maintenance of ES cells occurred with ESM at 37°C, 5% CO_2 by passing them when reaching 70% confluency on freshly thawn irradiated EF cells with a cell number of $2x10^6$. Cells were detached from the dish by washing twice with PBS and adding 1ml of 0.25% Trypsin/EDTA for 5min at 37°C followed by centrifugation at 290g for 5min and resuspension in fresh media. ESM was changed every day to keep appropriate concentrations of LIF and nutrients in the culture.

ES cells were also cultured without feeder cells. In this case they were directly thawn and cultivated on gelatinized 60mm dishes with F-ESM at 37°C, 5% CO_2. To get ES cells free of EF cells the mixture was incubated for 30min at 37°C. ES cells were aspirated and replated on gelatinized dishes.

5.3.8 Generating BAC-transgenic ES cell lines

To generate transgenic ES cell lines the cells were used under EF-free conditions because EF cells are not antibiotic-resistent.

$3x10^5$ ES cells were seeded per well of gelatinised 6 well plates. The next day cells were washed ones with PBS and 2ml OptiMEM were added.

140µl OptiMEM and 10µl Lipofectamine 2000 and 130µl OptiMEM and 20µl BAC DNA (concentration approximately 100ng/µl) were mixed each in one well of a 96 well plate followed by 5min incubation at room temperature. The two solutions were mixed and incubated for additional 20min at room temperature. The solution was added to the cells following incubation at 37°C, 5% CO_2 for 5h. Afterwards media was changed to F-ESM. The next day selection was started by changing culture media to F-ESM containing appropriate antibiotic concentration. Media was changed every day.

5.3.9 Establishment of ES cell clones

To establish single clones of the transfected, antibiotic selected ES cells 24 well plates were gelatinised and colonies that were derived from a single cell were picked. Therefore, 50µl 0.25% Trypsin/EDTA was prepared in 96 well plates, the colony was scratched and aspirated from the dish with a pipette adjusted to 10µl and transferred into the Trypsin. The cells were incubated for 5min at RT, transferred into F-ESM and cultured on gelatinised 24 well plates in 0.5ml F-ESM containing the appropriate antibiotic. The media was changed every day and as soon as the cells reach 70% confluency they were passed into 6-well plates and further grown up.

5.3.10 Differentiation of ES cells

To differentiate ES cells into lymphoid cells undifferentiated ES cells were washed twice with PBS and detached from the dish by addition of 1ml 0.25% Trypsin/EDTA for 5min at 37°C. The reaction was stopped by adding 4ml of and cells were centrifuges at 290g for 5min. After resuspending cells were counted and $0.4x10^4$ ES cells per well of a 6-well plate were seeded on 100% confluent OP9 stromal cells in 2ml αMEM/20% FCS (day 0). On day 3 of the culture 1ml of media was aspirated and 1ml of fresh media added (half media change). On day 5 the cells were passed by washing twice with PBS and incubation with 1ml 0.25% Trypsin/EDTA for 5min at 37°C. 1ml of αMEM/20% FCS was added and cells were vigorously pipetted to achieve a single cell suspension. To remove cell clumps cells were

filtrated through a 40µm mesh. The suspension was centrifuged at 290g for 5min and cells were resuspended in fresh media. $3x10^5$ cells per T25 culture flask were replated on fresh 100% confluent OP9 stromal cells in 5ml media. From day 5 to day 10 cells were cultured in αMEM/20% FCS supplemented with 3% SCF and 2% Flt-3L. On day 8 of the culture 2ml media were removed and 3ml fresh media added.

On day 10 cells were tapped off the stromal cells, aspirated and filtrated through a 40µm mesh. After centrifugation cells were resuspended and $1.1x10^5$ cells were seeded per well of a 6 well plate containing 100% confluent OP9 stromal cells for B lineage differentiation. Cells were continuously cultivated with 2ml/well αMEM/20% FCS containing 3% SCF and 2% Flt-3L. To change the culture conditions on day 12 whole media was aspirated and 2ml of fresh IMDM/2% FCS/0.03% Primatone containing 1% IL7 and 2% Flt-3L was added. On day 15 cells were detached from the dish by vigorous pipetting and filtrated through a 40µm mesh. Cells were centrifuged, resuspended and $1.1x10^5$ cells per well of a 6 well plate were replated on semi-confluent OP9 stromal cells. Culture conditions were continued as before. On day 19 cells were harvested by pipetting as on day 15 and analysed by FACS.

For T lineage differentiation cells were differentiated as described before until day 10. From day 10 on all media remained identical but stromal cells were changed to OP9-DL1.

To induce NK cells, cells from culture day 10 were cultivated on 100% confluent OP9 with αMEM containing 20% FCS, 3% IL-2 and 10ng/ml IL-15 for additional 8 days. Cells were harvested for FACS analysis and RNA preparation.

Erythrocytes were induced by cultivating cells from day 10 on confluent OP9 with αMEM/ 20% FCS/ 3% SCF/ 2U/ml Erythropoietin for additional 6 days. The cells were harvested for FACS analysis and RNA preparation.

To differentiate cells into osteoclasts, cells from day 5 were kept on OP9 cells without additional cytokines until day 10 and were subsequently passed to confluent layers of ST2 cells in αMEM/10% FCS supplemented with 10^{-8}M 1α,25$(OH)_2$D3 and 10^{-7}M dexamethasone for six additional days at $1x10^3$ cells/well on 24-well-plates [157]. Cells were analysed by TRAP staining.

5.3.11 In vitro evaluation of B- and T-lymphoid potential by OP9/OP9-DL1 coculture

For evaluating B-cell and T-cell potential, cells were single-cell sorted directly into 96-well plates containing preplated OP9 or OP9-DL1 cells (irradiated, semi-confluent) in IMDM/2% FCS/0.03% Primatone supplemented with 2% Flt-3L and 1% IL-7, respectively. For supplementation with cytokines recombinant cytokines (10ng/ml Flt-3L, 10ng/ml IL-7) were

also used instead of hybridoma cell culture supernatants. After 10 days cocultures were evaluated by microscopic appearance of colonies and flow cytometry analysis. Wells containing colonies with more than 100 cells were counted as positive.

5.3.12 Transient transfection of Platinum-E (Plat-E) cells

For transient packaging of retroviruses the Plat-E packaging cell line was used which is based on the 293T cell line carrying packaging constructs. Thereby the viral structural genes gag-pol and env are expressed under the EF1α promoter. (Morita et al. 2000)

To produce retroviruses $6x10^5$ cells were seeded per well of a 6-well-plate in DMEM/10%FCS and cultured overnight at 10% CO2, 37°C. The next day Plat-E cells were transfected by using Lipofectamin as cationic lipid. On a round bottom 96-well plate, 2µg DNA and 10µl Lipofectamin were each prediluted in 100µl DMEM, mixed together and incubated at room temperature for 20 minutes. In the meanwhile Plat-E cells were carefully washed twice with DMEM. Then they were covered with 800µl DMEM. The incubated DNA-lipid-mix was added to the cells and incubated for 5 hours at 10% CO2, and 37°C. Afterwards the media was replaced by 2 ml DMEM/10%FCS and the cells subsequently incubated at 10% CO2 and 37°C over night. After 24 hours the media was changed to fresh DMEM/10% FCS.

Two days later the supernatant including the desired virus was harvested from the cells, and centrifuged 1min at 10500g to pellet remaining Plat-E cells. The cell-free retroviral supernatant was either used directly or kept for a maximum of 3days at 4°C until usage.

5.3.13 Transduction of mammalian cells with viral vectors

The cells that were about to be transduced, were harvested and concentrated to $2x10^5$ cells in less than 100µl and transferred to a 2ml microcentrifuge tube. 500µl of the viral supernatant was added to the cells and the cells became spin-infected by centrifugation for 4h at 1100g, 30°C. Afterwards the supernatant was aspirated, the cells were resuspended in the adequate medium and cultured under normal conditions. If the vector carries a resistance gene transduced cell were drug-selected by addition of the appropriate antibiotic 24h after transduction.

5.3.14 Alkaline phosphatase staining

The staining was done under non sterile conditions. The medium was aspirated, 1ml 3.7% Formaldehyde/PBS per well of a 6-well plate was added and incubated for 10min at RT. During that time the Fast red violet LB salt was added to the alkaline phophatase staining solution (see 5.1.6.) The Formaldehyde was aspirated and the cells were washed twice with PBS. Per well 500µl alkaline phosphatase staining solution was added and incubated for 5-15min. The staining solution was aspirated and PBS was added.

5.3.15 TRAP staining

The staining was done under non sterile conditions. The medium was aspirated, 0.5ml 3.7% Formaldehyde/PBS per well of a 24-well plate was added and incubated for 10min at RT. During that time the Fast red violet LB salt was added to the TRAP staining solution (see 5.1.6). The Formaldehyde was aspirated and the cells were washed twice with PBS. Per well 500µl 50% Ethanol/50% Acetone (v/v) was added and incubated for 1min at RT. The plate was washed under tap water until no acetone fumes were detectable. Per well 250µl TRAP staining solution was added and incubated for 3-5min. The staining solution was aspirated and the plate was soaked in tap water for 30min.

5.4 Immunological methods

5.4.1 Fluorescence activated cell sorting (FACS)

Flow cytometry refers to the measurement of the physical and chemical characteristics of cells. A single-cell suspension is forced up by a slight overpressure relative to the sheath fluid creating a tiny stream of fluid taking the cells past the laser light one cell at a time. Single cells are analysed by their characteristic light scattering and their emitted fluorescence. The forward light scatter (FSC) / side light scatter (SSC) characteristics enables to distinguish between different cell populations by their size and granularity. Analysis of specific intracellular and extracellular marker antigens can be performed by staining with fluorescence dye coupled antibodies. The dyes are excited by appropriate laser and subsequently emit light of different wave lengths. This emission is measured by defined detectors.

All antibodies used in FACS stainings were titrated on cells expressing the appropriate marker for the optimal concentration of usage. In general 1:50, 1:100, 1:200, 1:400, 1:800, 1:1000

and 1:2000 dilutions of the antibody solution in FACS buffer were prepared. Antibody concentrations determined as optimal were used for all further stainings.

For multi-color stainings, up to six fluorescence-coupled antibodies were diluted together in FACS buffer. If a biotinylated antibody was included in this mixture its labelling occurred in a secondary staining procedure by subsequent addition of likewise diluted fluorescence-coupled streptavidin. For each cell type and measurement an unstained control was used which was treated as the stained sample but without antibody.

Per staining $5x10^5$ cells were transferred into a 96 well plate and centrifuged at 290g for 5 min. Per sample, 50µl of the FACS blocking solution (heat-inactivated rabbit serum 1:3 diluted in FACS buffer) was used and the samples incubated for 10 minutes. The suspension was centrifuged (290g, 5 min) and the supernatant discarded. Cells were resuspended in 50µl antibody solution and incubated for 20min light-protected on ice. The cells were centrifuged again and washed twice with 200µl FACS buffer. If required, the cells were resuspended in 50µl secondary staining solution, incubated and washed twice as described above. To distinguish between living and dead cells they were further stained with DAPI (1:1000) in FACS buffer for 5min, centrifuges and finally resuspended in 200µl FACS buffer and transferred into FACS tubes for measurement.

Stained cell suspensions were analysed on an LSRII flow cytometer. Unstained and single colour stainings were used to manually adjust PMT voltages. To remove signals which overlap in their emission spectra, compensation was performed using Compensation Beads in combination with the automatic compensation option of the FACSDiVa software. Data were summarized by using the FACSDiVa or FlowJo software.

5.4.2 FACS sorting

Sorting was performed by Toralf Kaiser (FACS facility MPI for Infection Biology, Berlin) using a FACSAria. After each sort a small fraction of the sorted cells was reanalysed to confirm its purity.

For single cell sorting with subsequent cultivation, populations were first pre-sorted into FACS tubes. During secondary sorting the cells were directly plated into 96-well plates containing the appropriate media.

5.4.3 ELISA

ELISA plates were coated with 50µl unlabeled antibodies against mouse IgM, IgG or IgA, diluted 1:500 in PBS. After one hour of incubation at room temperature the wells were washed three times with 200 µl PBS-T. If necessary the serum was prediluted, but at least 1:3 diluted in 3% BSA/PBS-T. Wild-type C57Bl/6 serum was used as standard and $Rag^{-/-}$ serum as negative control (blank). 60µl of the diluted serum were applied per well and incubated for one hour. The wells were washed three times with PBS-T. Bound serum antibodies were detected by incubation with 50µl 1:1000 diluted anti-IgM, -IgG or -IgA alkaline phosphatase-labeled antibody per well for one hours at RT. The wells were washed three times with PBS-T followed by addition of 50µl pNPP, diluted in 1M diethanolamin, pH 9.8. Emitted light was measured at 405nm with an ELISA reader 20-30min after substrate addition. The absorbance of the serum samples was calculated relative to the wild-type serum.

5.5 Animal work

All experiments were in compliance with the German animal protection laws in a protocol approved by the Landesamt für Gesundheit und Soziales, Berlin (G0099/08).

5.5.1 Transgenesis

Superovulation, harvesting of fertilized oocytes, pronuclear injections of the BACs and oocyte transfer into pseudo-pregnant female mice were done by Dr. Uwe Klemm and Karin Bordasch (Animal facility, MPI for Infection biology)

5.5.2 Transplantation (adoptive transfer)

Before transfer, cell suspensions were passed through a 40µm cell strainer to remove cell clumps. Cultured cells were washed twice with PBS to remove FCS. $5x10^6$ to $1x10^7$ cells were resuspended in a total volume of 100µl PBS per recipient mouse.

One day (22 to 26 hours) before the transfer, immunodeficient $Rag2^{-/-}$ $\gamma_C^{-/-}$ recipient mice were sub-lethally irradiated with 400rad.

Immediately before transfer, recipient mice were kept under infrared light for 5min. The animals were then transferred into a holding device, the tail was cleaned with 70% ethanol and the cells were injected into the lateral tail vein using a 0.45mm 26G needle.

5.5.3 Bleeding

Before bleeding, mice were kept under infra-red light for 5min to enhance blood circulation. The animals were then transferred into a holder device and a transverse incision through the major tail vein was made (the major tail vein was transversely injured). About 100µl blood were collected in a 1.5ml eppendorf tube. Blood was coagulated and subsequently centrifuged at 2150g to obtain serum (containing all soluble antibodies). The serum was separated from the lower cellular phase. It was transferred into a new tube and frozen at –20ºC until further analysis by ELISA.

5.5.4 Isolation and preparation of mouse cells for analysis

If needed blood was taken as described above before death.

Mice were sacrificed by cervical dislocation. Dead mice were desinfected with 70% ethanol and fixed with needles at arms on a cork plate. To open up the body, the skin was cut along the ventral anterior-posterior axis.

To isolate lymphocytes from the peritoneal cavity the peritoneum was opened by an incision along the anterior-posterior body axis. Cells were collected by peritoneal lavage with a Pasteur pipette and about 3 to 5 ml of FACS buffer.

The spleen was dissected after cutting the peritoneum along the ventral anterior-posterior body axis and transferred into 15ml polypropylene tubes containing 5ml FACS buffer. For isolation of the thymus the thorax was opened along the ventral anterior-posterior body axis with two parallel cuts to the left and to the right of the sternum from the diaphragm up to the head. The thymus lies directly or ventrally to the heart. It was dissected and transferred into a 15ml polypropylene tubes containing 5ml FACS buffer.

To dispense the cells, the organ was pushed through a metal sieve with a syringe plunger to prepare single cell suspensions. The cell suspension was collected into a polypropylene tube.

For collection of bone marrow one femur and tibia of each animal were used. After removal of all muscles from the bones, the hip and knee joints were disconnected. The bones were cut at one end so that the marrow became accessible. The bone marrow was flushed with 2ml FACS buffer by inserting a 0.45mm 26G needle into the marrow cavity.

Isolated organs and cells, as well as all solutions used, were permanently kept on ice or at 4ºC if not otherwise indicated.

5.5.5 Removal of erythrocytes

Mouse erythrocytes lack strong cell membranes and, consequently, swell and finally burst during incubation in this hypotonic solution; other cells remained intact. Spleen and blood cells containing high numbers of erythrocytes were centrifuged at 290g for 5min at 4º. The supernatant was removed and erythrocytes were eliminated by resuspension in 1ml RBC lysis buffer with subsequent incubation for 3min on ice. To stop the reaction 9ml of FACS Buffer were added and the cell suspensions were centrifuged at 290g for 5 minutes. The cellular debris enclosed in the supernatant was again removed and the cells were resuspended in 5ml FACS buffer.

6 Results

6.1 Establishment to develop ES cells into hematopoietic lineages *in vitro*

6.1.1 Establishment of a culture system to develop preB cells from mouse embryonic stem cells in vitro

Based on published data [138, 144, 145, 147] and experiences with the culture of preB cells from fetal liver and adult bone marrow an ES cell culture system to induce preB and preT cells has been established. Undifferentiated J1 ES cells were cultured in αMEM/20% FCS for 5 days on OP9 stromal cells to form mesodermal-like cells. Following trypsinisation single cell suspensions were filtrated to remove remaining OP9 stromal cell layers and seeded onto fresh confluent OP9 layers. Cells were cultured for 3 days with addition of Flt-3L as described by Cho et al. [145]. On day 8 of the culture cells were tapped off and transferred to fresh confluent OP9 stromal cell layers and cultured until day 12 in αMEM/20% FCS supplemented with IL-7 and Flt-3L. From day 16 on cells were thought to be preB cells which could be detected by expression of the B cell markers B220 and CD19 by FACS analysis [144, 145]. In our case preB cells could never be detected after following this differentiation schedule (Figure 6-1A). The number of differentiated ES cells on day 8 of the culture was very low and when the cells were passed at this stage they did not continue growing.

As SCF is known to be a proliferation inducing cytokine [140] it was added to the culture from day 5 to day 8 (Figure 6-1B). The proliferation during that time increased but the number of cells on day 8 was still too low and it seemed to be too early to induce lymphocytic development. Again, no preB cells were detected on day 19 of the culture.

Therefore, the time to develop hematopoietic cells from mesodermal-like cells of day 5 was prolonged until day 10 (Figure 6-1C). The culture was successful, but only low numbers of preB cells could be detected following the protocol. It seemed not to be advantageous passing cells on day 10 of the culture and changing the culture condition at the same time. Cells did not continue growing in this media. Therefore, cells were passed on day 10 and again cultured for 2 days in αMEM/20% FCS supplemented with SCF and Flt-3L (Figure 6-1D). The media was changed to αMEM/20% FCS supplemented with IL-7 and Flt-3L on day 12 without passage. Here, more preB cells than with any other condition so far tested were derived in the culture. If only SCF was added from day 5 to day 12 cells did not develop into preB cells after 7 additional days in media containing IL-7 and Flt-3L (Figure 6-1E). Due to experiences in the lab with the culture and maintenance of preB cells from fetal liver and bone marrow in

which IMDM/2% FCS/0.03% Primatone [190] supplemented with IL-7 is used, ES cell derived hematopoietic cells from day 12 were also cultured under those conditions (Figure 6-1F and G). IMDM media usage from day 12 on increased the efficiency of preB cell development of day 12 hematopoietic cells. For all further experiments the schedule as seen in Figure 1G is used for the differentiation of ES cells into preB cells.

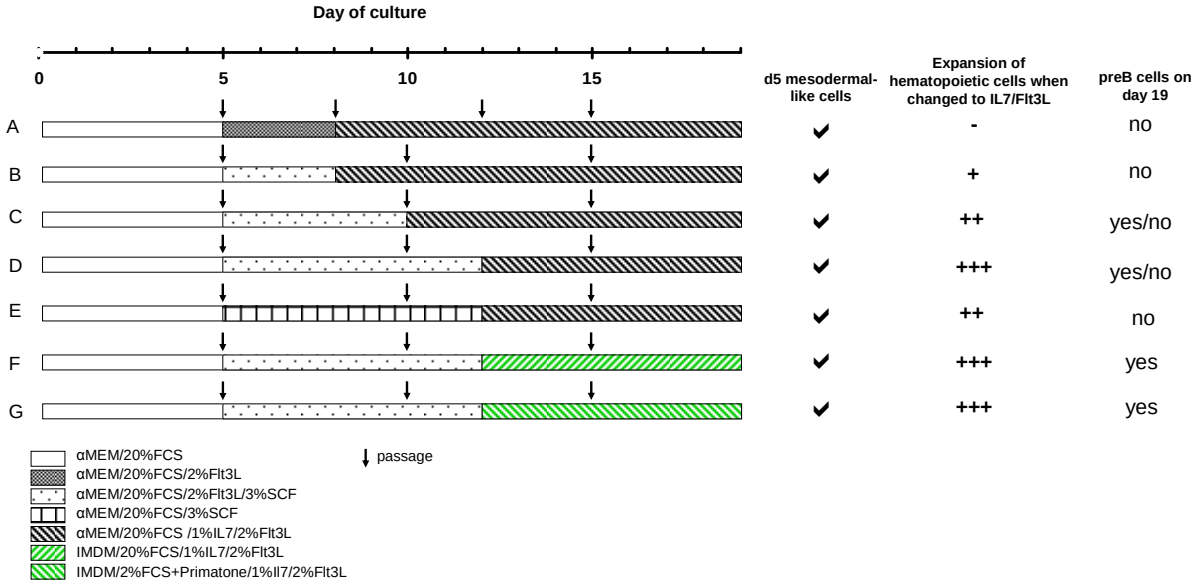

Figure 6-1: *In vitro* culture system for the development of ES cells into preB cells. Different conditions were tested and analysed for there potential to induce preB cells from ES cells.

Figure 6-2 shows the morphologic appearance and expression of lineage specific markers of mesodermal-like cells from day 5 of the culture, hematopoietic progenitor cells from day 10 of the culture and preB cells from day 19 of the culture. By day 5 of the culture most differentiating cells express Flk-1 indicating mesodermal development [46, 47], some also express CD41, as a very early marker of hematopoiesis [48, 49]. At day 10 of the culture the expression of Flk-1 is downregulated whereas the cells express the hematopoietic marker CD45 [45, 51-54]. In the case of successful B lineage differentiation the cells express the markers B220 and CD19 [191, 192].

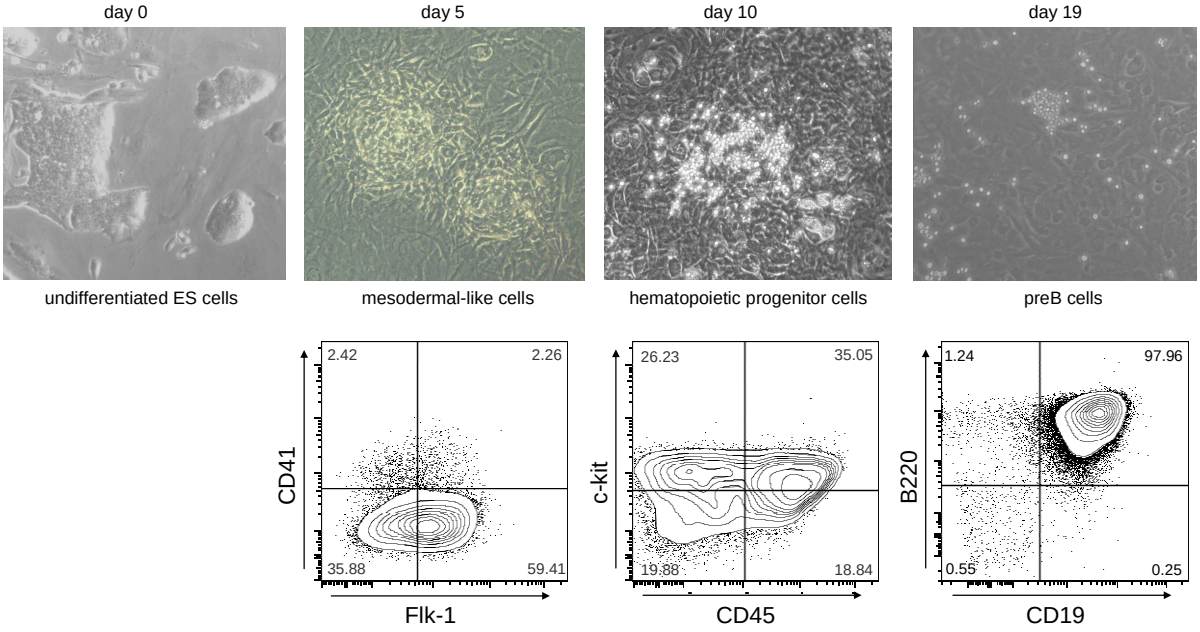

Figure 6-2: Morphologic appearance and expression of surface markers during development of preB cells from ES cells *in vitro*.

Growth curves from 13 independent differentiation experiments with the J1 ES cell line show that the total numbers of differentiating cells in the culture vary by a factor of 100 (Figure 6-3A). Two additional ES cell lines were tested and compared to J1 ES cells for their *in vitro* B cell differentiation potential. All three cell lines give rise to similar percentages of preB cells on day 19 of the culture ranging from 50% to 95%. The two 129 derived cell lines (J1 ES, GK3 ES) show better proliferation as seen by the higher total number of differentiating cells in the culture (Figure 6-3B). The preB cells ($B220^+$ $CD19^+$) could be kept in culture on OP9 stromal cells and IMDM/2% FCS/0.03% Primatone/1% IL-7 for at least 20 additional days.

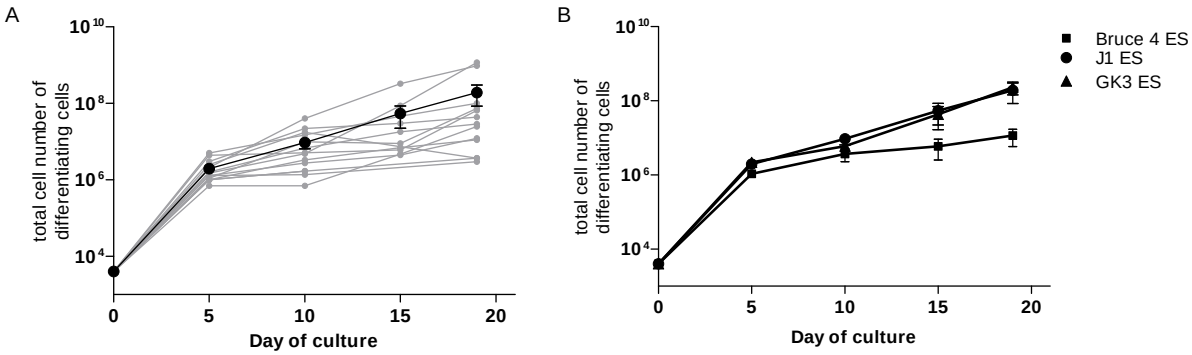

Figure 6-3: Growth curve of J1 ES cells from 13 independent experiments (black: mean±standard deviation) (A) and compared to two ES cell lines (Bruce4 ES and GK3 ES) (B). Dots show mean±SD.

6.1.2 B cell maturation of ES cell derived preB cells

To induce maturation of preB cells IL-7 and OP9 stromal cells, the two factors which keep preB cells in its developmental stage, were removed. ES cell derived preB cells were harvested and subcultured in the absence of IL-7 and stromal cells, but in the presence of IL-4, IL-5 and αCD40 for 4 days. The cell number decreased drastically whereby approximately 30% of the surviving cells expressed sIgM, a few of those also sIgD (Figure 6-4).

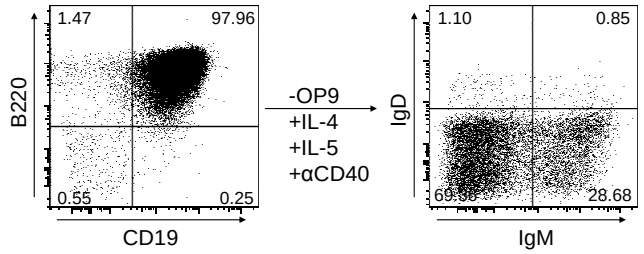

Figure 6-4: Surface marker expression of mature B cells after induction with IL-4, IL-5 and αCD40 for 4 days.

6.1.3 Development of preT cells from ES cells in vitro

Based on the culture system that was developed to induce preB cells *in vitro* from ES cells, the conditions were changed at the stage of the appearance of hematopoietic cells (day 10). The cells were passed to OP9-DL1 stromal cells to provide Notch signalling which is needed for T cell development in media containing SCF and Flt-3L. 2 days later, the media was changed to IMDM/2% FCS/0.03% Primatone supplemented with IL-7 and Flt-3L (Figure 6-5A).

On day 19 of the culture a mixture of preT and T cells derived containing $CD4^-$ $CD8^-$ double negative cells as well as $CD4^+$ $CD8^+$ double positive and $CD4^+$ or $CD8^+$ single positive cells (Figure 6-5B). Due to progressive cell death preT cell lines could not be established from those cells.

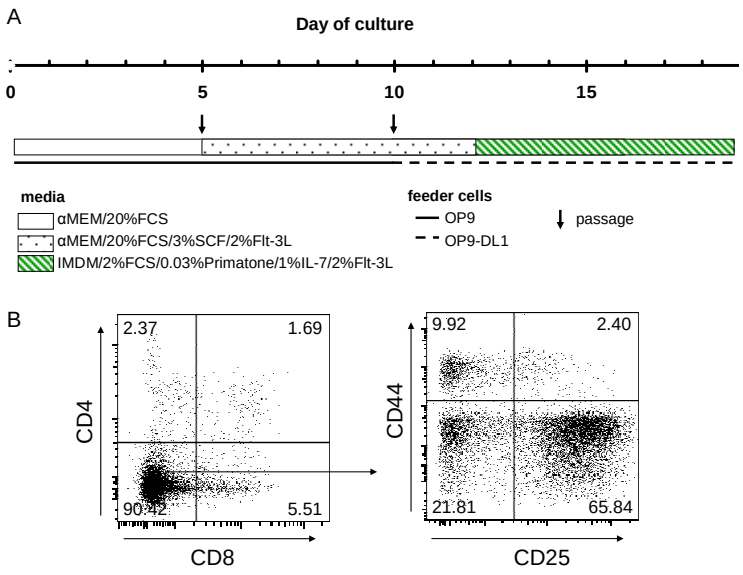

Figure 6-5: *In vitro* culture system to induce T cell development from ES cells (A) and surface marker expression of cells on day 19 (B).

6.1.4 Development of NK cells from ES cell in vitro

NK cell development was induced from day 10 on OP9 in the presence of IL-2 and IL-15 (Figure 6-6A). Eight to 10 days after induction NKG2A/E/G$^+$ natural killer cells developed in the respective cultures (Figure 6-6B). Granzyme A and perforin are enzymes known to be expressed and involved in the killing process in NK cells [193]. To analyse whether the differentiated cells express genes which are characteristic for the functional properties of NK cells RT-PCR analyses were performed to test for expression of granzyme A and perforin (Figure 6-6B).

The NK cells could not be maintained in culture. They killed the OP9 stromal cell layer (data not shown). This confirms the functional characteristics of the developed NK cells.

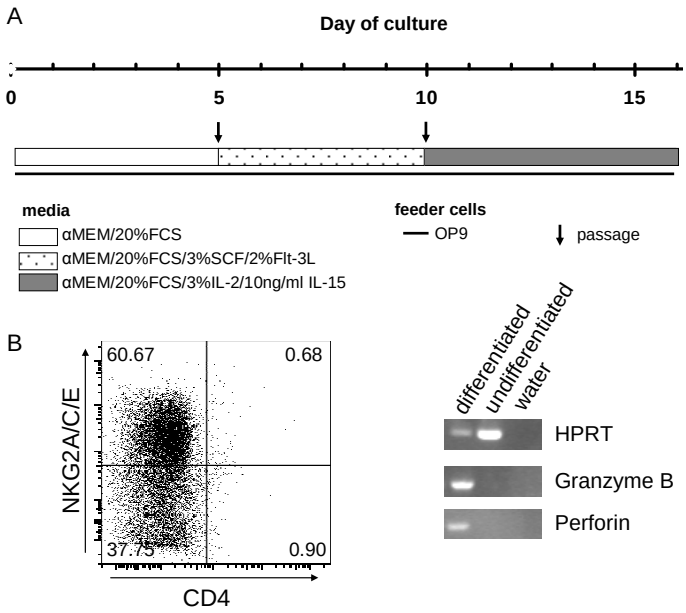

Figure 6-6: ***In vitro*** **culture system to induce NK cell development from ES cells (A) and surface marker expression of cells on day 16 (B).**

6.1.5 Development of erythrocytes from ES cells in vitro

Erythropoiesis was induced by culturing the day 10 differentiated ES-derived hematopoietic progenitors in the presence of SCF and erythropoietin (Figure 6-7A). On day 18 CD71$^+$ and Ter119$^+$ cells were detected (Figure 6-7B). To test for the expression of fetal (ζ) and adult (β) type-globin RT-PCR was performed. Data in Figure 6-7B show that both types of globin were expressed in the CD71$^+$ Ter119$^+$ cells, indicating that both primitive and definitive hematopoiesis had been induced in these cultures.

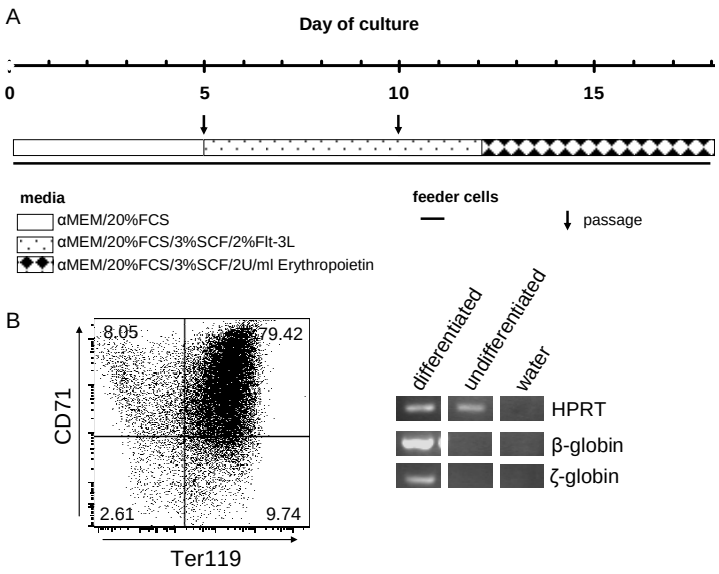

Figure 6-7: *In vitro* culture system to induce erythrocyte development from ES cells (A) and surface marker expression and globin RNA detection of cells on day 18 (B).

6.1.6 Osteoclastogenesis from ES cells

To set up the differentiation culture to develop osteoclasts, the protocol developed by Yamane et al. was followed [158] (Figure 6-8A). ES cells were seeded on OP9 stromal cells in the same way as to develop lymphoid cells. Mesodermal-like cells from day 5 were further expanded for additional 5 days on OP9 in SCF-containing media and then seeded on ST2 stromal cells, which are not deficient for M-CSF. Furthermore 1α,25-dihydroxyvitamin D_3 and dexamethasone were added to the culture to provide RANKL expression on the stromal cells. After 6 days in that conditions the cells developed into mature osteoclasts which were evaluated by TRAP staining (Figure 6-8B).

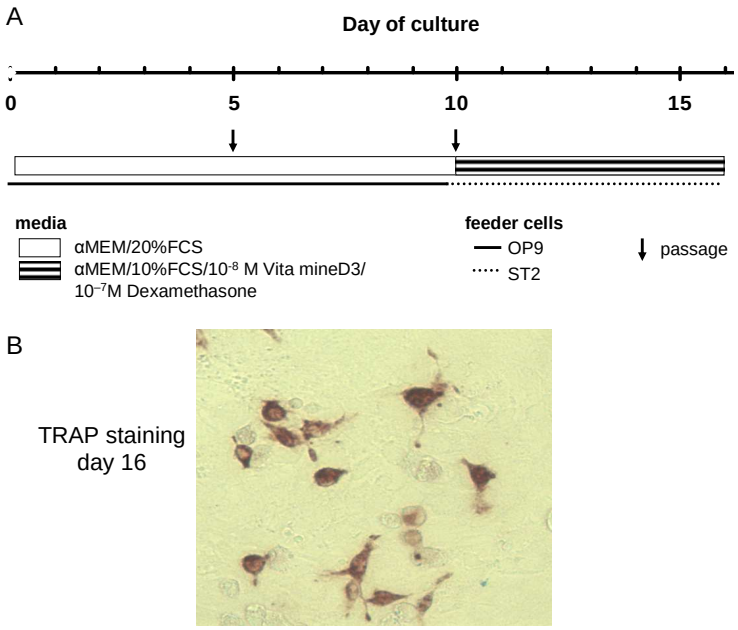

Figure 6-8: *In vitro* culture system to induce osteoclast development from ES cells (A) and TRAP staining of cells on day 16 (B).

6.2 Comparison of iPS and ES cells in terms of their efficiency to develop into hematopoietic lineage *in vitro*

The iPS cells used in this study were generated by Klaus Karjalainen, Nanyang Technological University of Singapore. The cells derive from BM of 5-Fluorouracil-treated C57Bl/6 mice (day 4) which were isolated and cultivated in the presence of the hematopoietic cytokines stem cell factor (SCF) and IL-6 [194]. The cells were induced to dedifferentiate by retroviral transduction with Sox-2, Oct-4 and Klf-4 as previously described [27]. All iPS cell clones derived from independently picked original colonies were tested for pluripotency markers along with the positive control ES cell line Bruce4. Alkaline phosphatase activity, SSEA-1 surface antigen level, Nanog and Oct3/4 expression were used as indicators for stem cell activity of the generated iPS cell clones [4, 10, 195]. Embryoid body (EB) formation and the ability to induce tumours are additional characteristics of ES-like stages [137, 196]. All iPS cell clones embloyed in this study are able to form EBs *in vitro* and large tumours after subcutaneous injection into $Rag2^{-/-}\gamma_c^{-/-}$ mice, suggesting that the iPS cells were capable of forming teratomas.

For the transduction with Sox-2, Oct-4 and Klf-4 the pMY [197] backbone which is known to produce high-titer retroviruses in transient transfection was used. In contrast to pMX vectors these viruses can efficiently express genes in most ES and hematopoietic progenitor cells [197].

6.2.1 iPS cells develop with reduced efficiency into lymphoid, erythroid and myeloid cells in vitro

Lymphopoiesis, erythropoiesis and myelopoiesis were induced from seven independent clones and were analysed in comparison to the control ES cell lines J1 ES (129 Sv derived) and Bruce4 ES (C57Bl/6 derived).

Development of preB cells from all seven iPS cell clones was severely reduced when compared with development from ES cells. iPS A generated approximately 10-fold, iPS B, C, and F between 1000- to 10 000-fold reduced numbers of cells, while iPS E and G generated no detectable numbers (i.e. at least 100 000 fold lower numbers) of B cells (Figure 6-9). Maturation could not be shown from iPS cell derived preB cells because of low numbers.

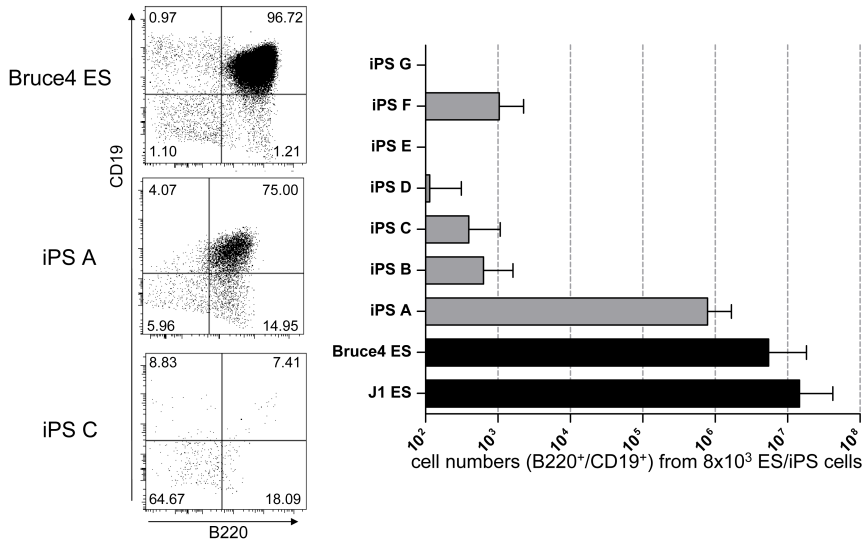

Figure 6-9: Surface marker staining of preB cells derived from ES and iPS cells, respectively. Graph shows numbers of $B220^+/CD19^+$ cells on day 19 of culture derived from $8x10^3$ cells seeded on day 0. Coumns represent mean±SD (N=6).

Developing T cells were subdivided into the different stages. In all stages the highest number of cells developed from both control ES cell lines. DN1 preT cells developed with comparable efficiencies from iPS A, B and F, all with only 5 to 10 times lower numbers than from ES cells. iPS C, D, E and G gave rise to 100 times lower numbers of DN1 cells compared to ES cells. The difference in the developed numbers of preT cells in the DN2, DN3 and DN4 stages were more drastic, i.e. iPS A, B and F gave rise to 50-100 times lower numbers, wherease iPS C, D, E and G showed reduced numbers by a factor 500-1000. Single and double positive T cells were reduced by a factor of 500-1000 for all iPS cell clones. In summary, all T cell developmental stages developed best from iPS A, B and F among all iPS cell clones, showing less reduction in all stages. (Figure 6-10).

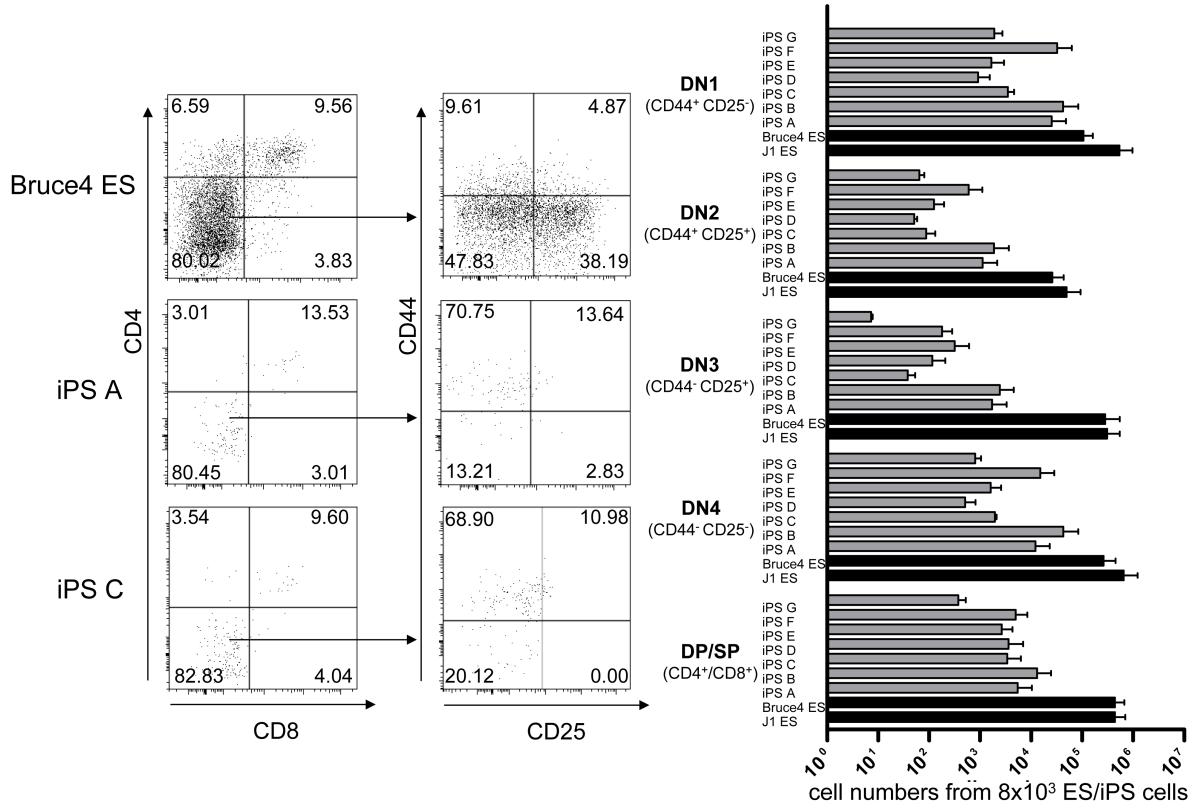

Figure 6-10: Surface marker staining of preT and T cells derived from ES and iPS cells, respectively. Graph shows numbers of cells expressing indicated markers on day 19 of culture derived from $8x10^3$ cells seeded on day 0. Coumns represent mean±SD (N=5).

NK cell development from C57BL/6 derived cells can be monitored by expression of NK1.1, therefore this marker was used to compare the efficiency of the iPS cells compared to Bruce4 ES cells.

All iPS cell clones developed NK cells, but with at least 10 times lower efficiency. NK cells from all iPS cell clones expressed perforin, and all but iPS G expressed granzyme A at a detectable level (Figure 6-11). Among the iPS cell clones the efficiency to develop $NK1.1^+$ cells was best for iPS A, B, C and D. iPS E was the clone showing lowest numbers of developed cells.

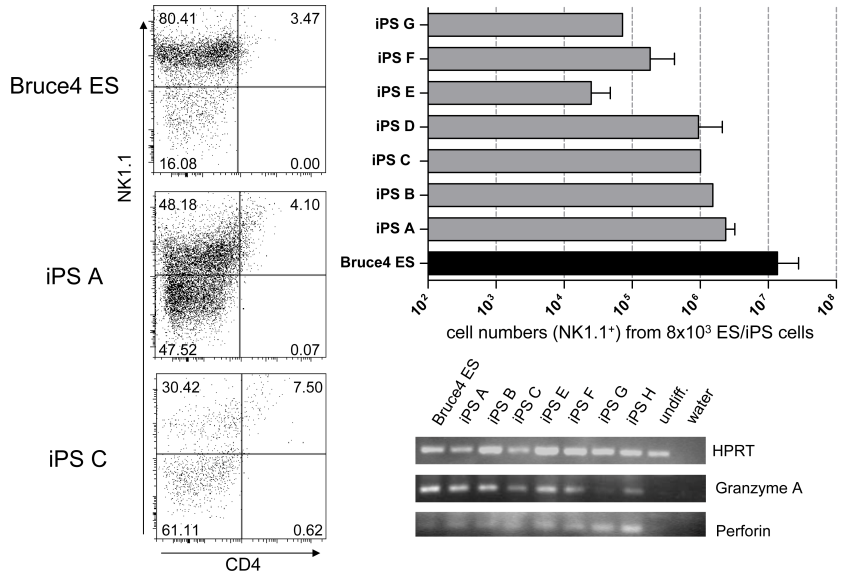

Figure 6-11: Surface marker staining of NK cells derived from ES and iPS cells, respectively. Graph shows numbers of NK1.1$^+$ cells on day 16 of culture derived from 8x10^3 cells seeded on day 0. RT-PCR was performed on Granzyme A and Perforin mRNA from cells harvested on day 16. Coumns represent mean±SD (N=3).

The iPS cell clones A and B developed a similar number of erythrocytes compared to ES cells, with evident expression of fetal ζ- and adult β-globin. In contrast, iPS C-G developed 100-1000 times lower numbers of erythrocytes with very low expression of fetal ζ-globin expression for iPS G and H (Figure 6-12).

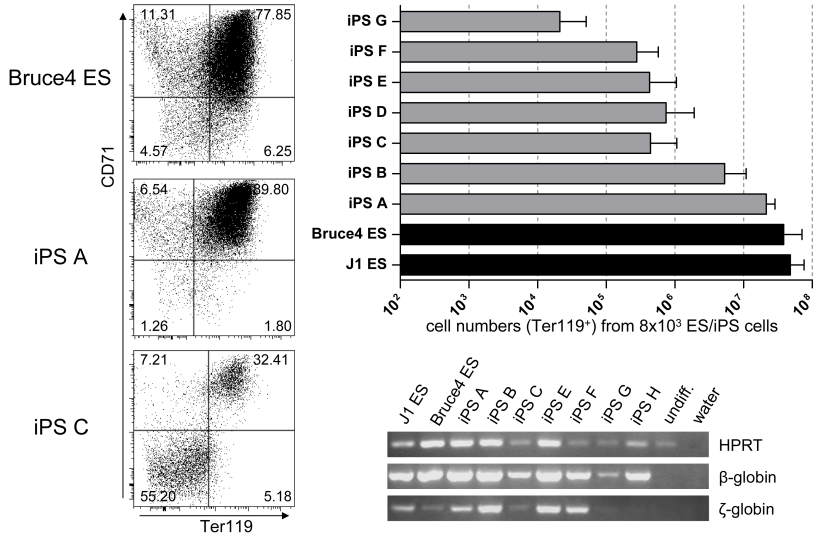

Figure 6-12: Surface marker staining of erythrocytes derived from ES and iPS cells, respectively. Graph shows numbers of Ter119$^+$ cells on day 18 of culture derived from 8x10^3 cells seeded on day 0. RT-PCR was performed on β- and ζ-globin mRNA from cells harvested on day 18. Coumns represent mean±SD (N=3).

Osteoclast induction of all iPS cell clones revealed that only iPS clones A, B, D, E and F developed detectable numbers of TRAP$^+$ osteoclasts. They were approximately 20-fold (iPS A) to more than 100-fold lower (iPS B, D, F) than the numbers of osteoclasts developed from normal ES cells (Figure 6-13).

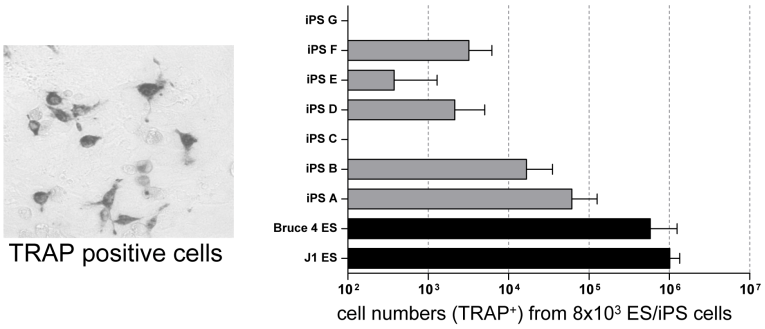

Figure 6-13: TRAP staining of osteoclasts derived from ES and iPS cells, respectively. Graph shows numbers of $TRAP^+$ cells on day 16 of culture derived from $8x10^3$ cells seeded on day 0. Coumns represent mean±SD (N=3).

All seven iPS clones developed several types of hematopoietic lineages, including lymphoid, erythroid and myeloid cells.

In summary, iPS A and B, were the most potent clones, while iPS clones E, D and G showed severe defects in the development of several hematopoietic lineages. It is evident from these experiments that each iPS clone had a characteristic pattern of severity in defective lineage differentiation. Among all iPS cells iPS A was best to differentiate into B, NK, erythroid and myeloid lineage cells. In all differentiation cultures iPS B was only slightly less effective, in T cell development even more effective than iPS A. iPS C, F and G among all iPS cell clones were at the average, but iPS C could not develop $TRAP^+$ osteoclasts. iPS E could not give rise to B cells, but developed a few T and NK cells and was not worse than others in differentiation to erythrocytes. iPS G was not able to develop into B lymphoid cells and myeloid osteoclasts, whereas T cells, NK cells and erythrocytes could be differentiated.

From these results it can be concluded that a series of iPS cell lines, all generated from bone marrow cells cultured under IL-6/SCF conditions, have different, but all of them reduced capacities to develop *in vitro* into myeloid, erythroid and lymphoid cell lineages.

To find the stage in which the reduced capacity manifests in the culture earlier phases in development were investigated.

6.2.2 Reduced capacity of hematopoietic differentiation of iPS cells becomes manifest during the development from mesodermal to hematopoietic progenitors

Within the first five days on OP9 cells the differentiating ES cells expanded by 100-200 fold, so that from $8x10^3$ ES cells $8x10^5$ to $2x10^5$ differentiated, mesodermal-like cells were generated (Figure 6-3). It is evident from the data in Figure 6-14 that the proliferative expansions of four iPS cell lines (iPS A, B, C, F) between day 0 and 5 after induction of differentiation were comparable to normal ES cell differentiation, i.e. generated 50-100 times the number of cells plated at day 0. Three iPS cell lines (iPS D, E, G) were around 20% less

efficient. Only iPS A continued proliferation until day 10, whereas all other iPS cell clones showed a drop in cell numbers during differentiation from day 5 to day 10.

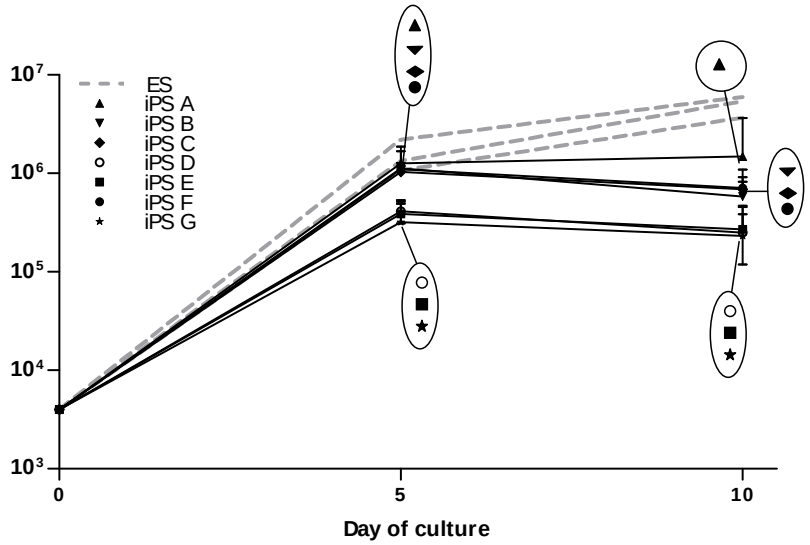

Figure 6-14: Growth curves from day 0 to day 10 of all iPS cell clones and three ES cell lines.

Like differentiated ES cells, approximately half of all the differentiated iPS cells of each clone expressed the mesodermal marker Flk-1 [46, 47] (Figure 6-15B). The cells developed from ES and iPS cells are not distinguishable by their morphological appearance on day 5 of the culture (Figure 6-15A). The percentage of Flk-1$^+$ cells on day 5 of the culture is comparable between the ES and iPS cells (Figure 6-15B). The expression of CD41, a marker of very early hematopoiesis [48-50] was around 2 to 5-fold lower in all iPS cell clones compared to ES cells at this stage (Figure 6-15B).

At day 5 differentiating cells were collected and replated on fresh confluent OP9 cells, supplemented with SCF and Flt-3 ligand (Flt-3L). In this second period of culture expression of Flk-1 was downregulated, while expression of CD45 was upregulated (Figure 6-15B). The differentiating ES cells expanded at best by a factor of 3. In experiments not shown here it was found that the Flk-1$^+$ cells which also expressed Tie-2 were the hematogenic mesodermal progenitors that developed into the CD45$^+$ cells [157, 198, 199].

Between day 5 and 10 all iPS cell lines developed less well than the normal ES cells evident in a lack of further proliferative expansion (Figure 6-14) and a concomitant lower number of CD45$^+$ cells in the cultures at day 10 (Figure 6-15B). However, the morphology of the cells on day 10 is not distinguishable between ES and iPS cells (Figure 6-15A).

At day 10 the proportion of cells expressing CD45 as a hematopoietic marker is drastically decreased in the iPS cell clones C-G, while the iPS cell clones A and B show similar percentages of CD45$^+$ cells in the culture compared to Bruce4 and J1 ES cells (Figure 6-15B).

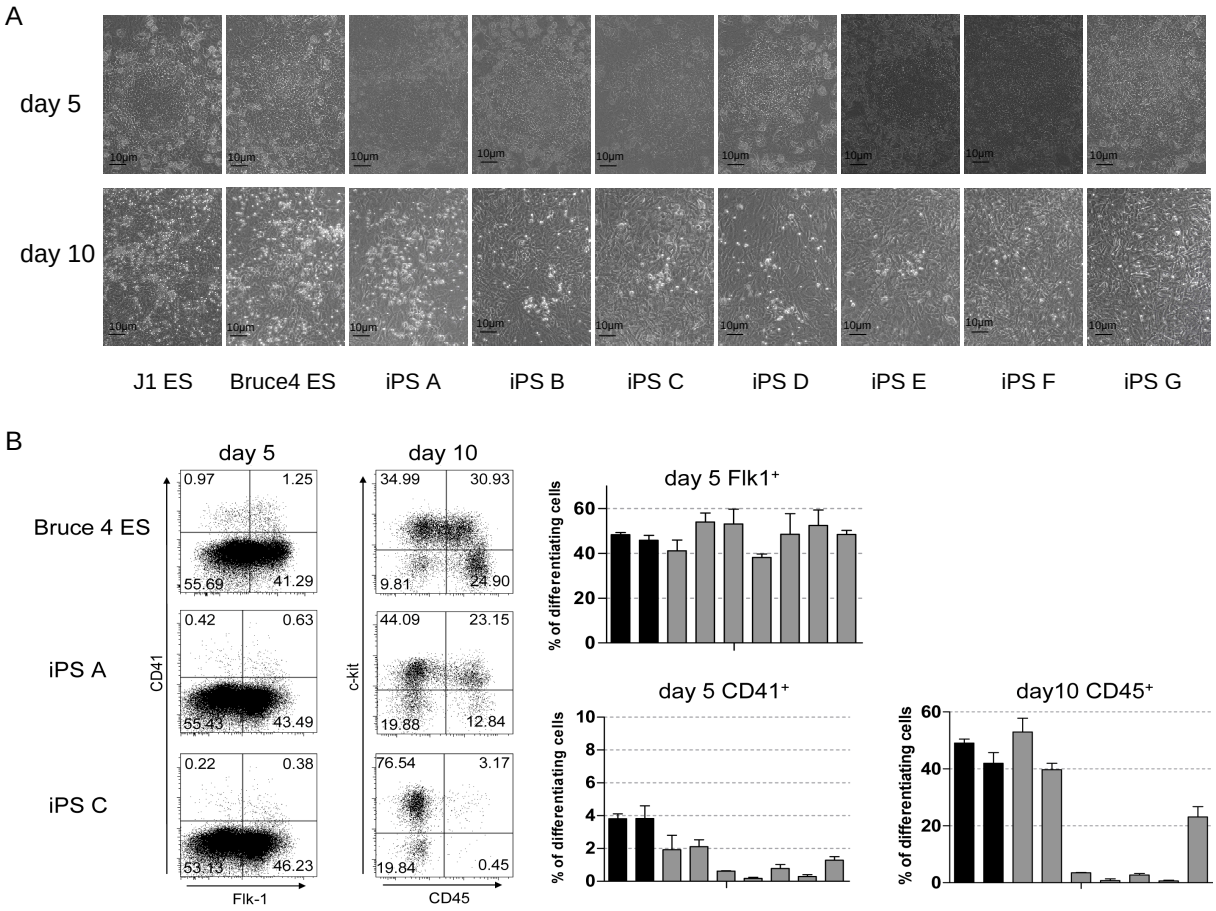

Figure 6-15: Morphologic appearance (A) and surface marker expression (B) of differentiating cells from day 5 and day 10. Graphs show percentages of cells expressing indicated surface markers.

6.2.3 Expression of Sox-2, Oct-4 and Klf-4 in undifferentiated and differentiating ES and iPS cells

One reason for the reduced potential of iPS cells to differentiate into hematopoietic lineages might be that the vectors that have been introduced into the cells to induce pluripotency are still active at high levels, even after iPS cells are induced to differentiate. This activity may inhibit their potential to differentiate.

Quantitative RT-PCR analyses were performed to determine the expression of Sox-2, Oct-4 and Klf-4 in ES cells and retrovirally transfected iPS cells (Figure 6-16). To distinguish between endogenous and exogenous expression qRT-PCR was also performed using primers that specifically amplify the endogenous genes by binding to the UTR sequences that are absent in the retroviral vectors. Nanog as a stem cell specific gene was also added as control. Normal ES cells express Sox-2, Oct-4 and Klf-4 in the undifferentiated stage at an expected level. When they differentiate into mesodermal-like cells from day 5 of the culture they downregulate the expression of those genes by a factor of 100 for Sox-2 and a factor of 5-10 for Oct-4 and Klf-4.

All seven iPS lines show very similar patterns of downregulation of Oct-4 and Kfl-4 from day 0 over day 5 to day 10 of induction of iPS differentiation, all at higher levels compared to the two ES cell lines (Figure 6-16A). Compared to ES cells iPS cells express 5000-10000 times more Klf-4 and Oct-4 in the undifferentiated stage. The expression levels of Oct-4 and Klf-4 are reduced in all iPS cell clones when the cell differentiate, but remain 100-1000-fold increased in differentiating iPS cells (day 5 and day 10).

Expression of Sox-2 is around 10-fold higher in all iPS lines, compared with ES cells. However, their capacity to downregulate Sox-2 is markedly different. iPS A and B express lower levels of Sox-2 on day 5 of the culture which are similar to those in differentiating ES cells, while iPS lines C, E, F, G and H do not downregulate Sox-2 expression to a comparable extent. In iPS lines C, D and G Sox-2 expression is even upregulated again on day 10 of differentiation (Figure 6-16A). In contrast to that the analysis of the endogenous gene expression shows a very similar pattern compared with ES cells in all three differentiation stages (Figure 6-16B).

The ES cell-like Sox-2 downregulation at day 5 in iPS lines A and B is in line with their higher efficiency to develop into hematopoietic lineages compared to the other iPS cell clones. It is also notable that the downregulation of Sox-2 expression in ES cells at day 10 of differentiation is not followed by the iPS lines A and B, a difference that may contribute to the lower hematogenic capacity of the iPS lines (Figure 6-16B).

From these results it appears that the pattern of Sox-2 expression in iPS cells during hematopoietic differentiation is a critical factor for the efficiency of this differentiation.

From the endogenous gene expression analyses of Sox-2, Klf-4, Oct-4 and Nanog it becomes clear that the ectopic overexpression of Sox-2, Klf-4 and Oct-4 itself has an effect on the iPS cells reducing their potential to differentiate into hematopoietic lineages, most pronounced for B lymphoid cells, erythrocytes and myeloid osteoclasts.

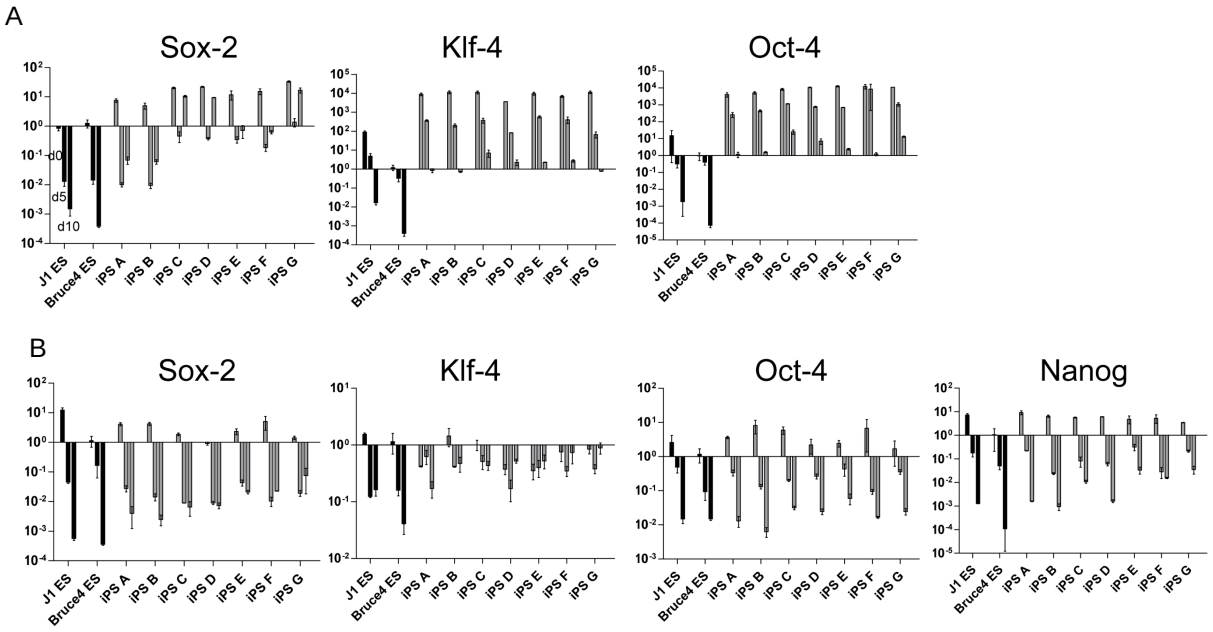

Figure 6-16 Total (A) and endogenous (B) RNA levels of Sox-2, Klf-4, Oct-4 and Nanog in undifferentiated (day 0) and differentiating (day 5 and 10) ES and iPS cells. (first column: day 0, second column: day 5, third column: day 10). The RNA levels shown normalized to GAPDH and relative to undifferentiated Bruce ES (day 0). Columns represent mean±SD (N=3).

6.3 Reconstitution of immunodeficient mice with hematopoietic progenitors developed from mouse ES cells *in vitro*

6.3.1 Differentiation of ES cells into hematopoietic progenitor cells

Following differentiation of ES cells into all kinds of hematopoietic lineages the cells have been shown to express the mesodermal marker Flk-1 on day 5 and the hematopoietic marker CD45 on day 10 (Figure 6-2). To get detailed information on the changes of surface marker, especially of mesodermal and hematopoietic markers, the cells from day 5 to day 12 were monitored daily for expression of the mesodermal marker Flk-1, the early hematopoietic marker, CD41, and of the hematopoietic marker CD45. All of those markers are not expressed on ES cells.

On day 5 of the culture Flk-1 was expressed on 60%, CD41 on around 5% and CD45 on less than 1% of the differentiating ES cells (Figure 6-17).

CD41 expression increased to a peak at day 8 when over 80% of the cells were $CD41^{+}$. Between day 6 and 7 after induction of differentiation CD45 began to be expressed on more than half of the cells. This indicated the development of hematopoietic cells in the cultures. Flk-1, on the other hand, was downregulated, so that at day 10 less than 10% and at day 12

less than 1% of the cells expressed Flk-1. Hence, it appeared that Flk-1^{+} mesodermal cells did not remain at later stages in these differentiating cultures (Figure 6-17).

Based on those data, which appear to parallel hematopoietic development *in vivo* in the embryo (discussed in detail in 7.3), it was expected that transplantable hematopoietic progenitors would be detectable in the culture between day 5 and day 12.

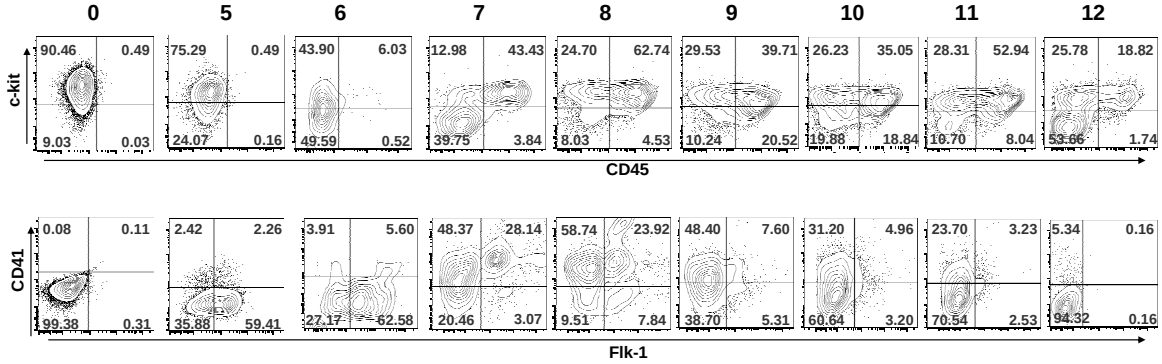

Figure 6-17: Differentiation of ES cells monitored by differential expression of Flk-1, CD41, CD45 and c-kit by FACS.

To study the *in vivo* lymphoid and myeloid repopulation potential of the *in vitro* differentiated cells 5x10^{6} cells from cultures at day 5, 6, 7, 8, 9, 10, 11 or 12 were transplanted into sublethally irradiated immunodeficient Rag2$^{-/-}$ $\gamma_c^{-/-}$ Balb/c mice (Figure 6-18). For each day of the culture at least four independent experiments with 3 transplanted mice each were performed.

To get first hints of B cell reconstitution in the transplanted host serum was taken after one and 4 month after transplantation and analysed for the presence of IgM and IgG. 4 months after transplantation serum IgM-positive mice were analysed by FACS for the presence of J1 ES derived lymphoid and myeloid cells in the bone marrow, thymus, spleen, peritoneal cavity and blood. J1 ES cell derived cells could be distinguished from the host cells by their different MHC I haplotypes (J1ES: H2K^{b}, Rag2$^{-/-}$ $\gamma_c^{-/-}$ Balb/c mice: H2K^{d}). As negative controls Rag2$^{-/-}$ $\gamma_c^{-/-}$ mice were sublethally irradiated but did not receive any cells. Transplantation of mice with wild-type C57BL/6 bone marrow cells served as positive controls.

All experiments were repeated with another ES cell line, GK3 ES, having GFP knocked into the ROSA26 locus. In that case Rag2$^{-/-}$ $\gamma_c^{-/-}$ C57Bl/6 mice were used. The ES cell-derived cells could be distinguished from the host cells by GFP expression.

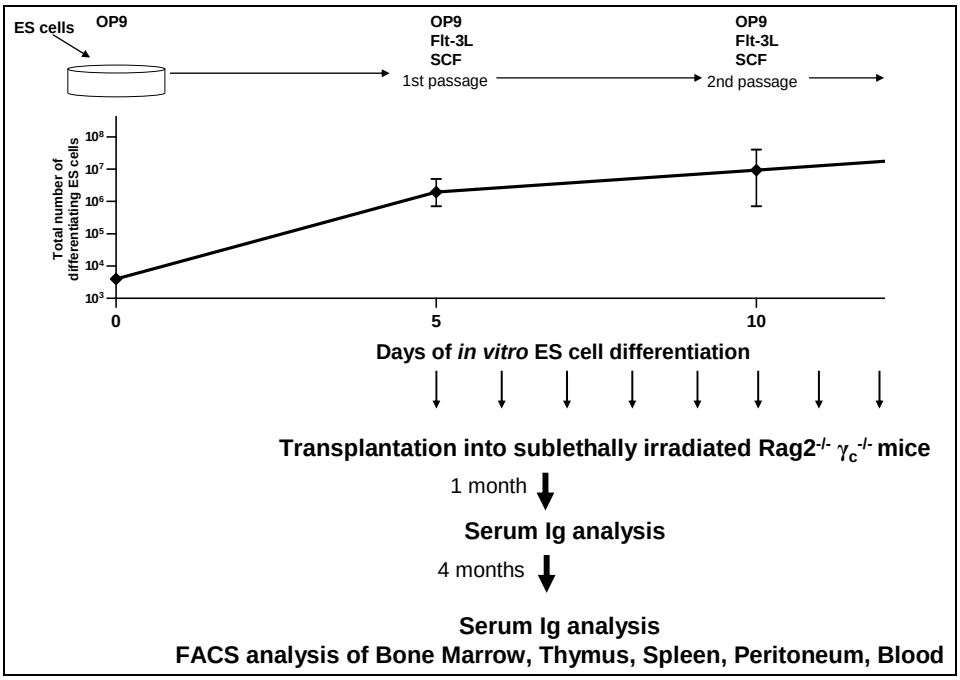

Figure 6-18: Transplantation of hematopoietic progenitors from the culture of *in vitro* differentiating ES cells. Arrows indicate the times of transplantations of $5x10^6$ cells into sublethally irradiated $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice.

6.3.2 Reconstitution of IgM and IgG in sera of $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice transplanted with differentiating ES cells.

First, development of B cells in the transplanted recipients was monitored one and 4 months after transplantation. Immunodeficient $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice cannot produce Ig themselves. Therefore, the presence of IgM and IgG in the sera of transplanted mice implies the development and presence of donor-derived Ig-secreting mature B cells.

Mice transplanted with normal BM cells ($5x10^6$ cells) always reached the same lvels of IgM and IgG (100%) in their sera as wild-type mice. In ccntrast, mice transplanted with cells from culture days 7, 8 and 12 did not have detectable levels of IgM or IgG in their sera one month after transplantation. In contrast, 55% to 60% of the mice transplanted with cells from day 9, 10 and 11 had detectable IgM levels in their sera, and 25% to 30% also had IgG (Table 6-1).

	Days of culture					
	7	**8**	**9**	**10**	**11**	**12**
	Number of mice with Ig in the serum **/** Total number of transplanted mice					
IgM	0/4	0/22	24/44 (55%)	17/28 (61%)	11/20 (55%)	0/3
IgG	0/4	0/22	13/44 (30%)	7/28 (25%)	6/20 (30%)	0/3

Table 6-1: Frequencies of mice receiving transplants of differented ES cells with detectable Ig in their sera. Sera of transplanted mice were assayed for presence of IgM and IgG by ELISA 4 months after transplantation. Levels of Ig below detection limit (IgM 0.06µg/ml; IgG 0.32µg/ml) are called negative.

The levels of serum IgM and IgG were comparable in mice transplanted with cells from day 9, 10 and 11 (Figure 6-19A and B). The levels of IgM reached, at best, between 1% and 6% of the levels found in the control mice transplanted with bone marrow cells (Figure 6-19A)

To follow whether B cell reconstitution occurs only for a short period of time or persists long-term serum IgM and IgG levels were compared between 1 and 4 months after transplantation in the serum of Ig-positive recipients. IgM levels did not change between 1 and 4 months after transplantation. IgG levels appeared to increase in some of the recipients, but the levels remained low, i.e. in the best cases around 10% of the control wild-type mouse serum (Figure 6-19B). Analyses done at 4 months after transplantation showed that recipients negative for Ig remained negative.

These data show that serum Ig was detectable in $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice from ES cells differentiated *in vitro* for 9, 10 and 11 days, but not from those differentiated for a shorter time, i.e. for 7 or 8 days *in vitro*, and not from later cells, i.e. those differentiated for 12 days (Figure 6-19A and B) or longer (not shown).

The results of differentiated J1ES cells transplanted into $Rag2^{-/-}$ $\gamma_c^{-/-}$ Balb/c mice are comparable to those obtained from GK3ES cells/ $Rag2^{-/-}$ $\gamma_c^{-/-}$ C57BL/6 mice transplantations.

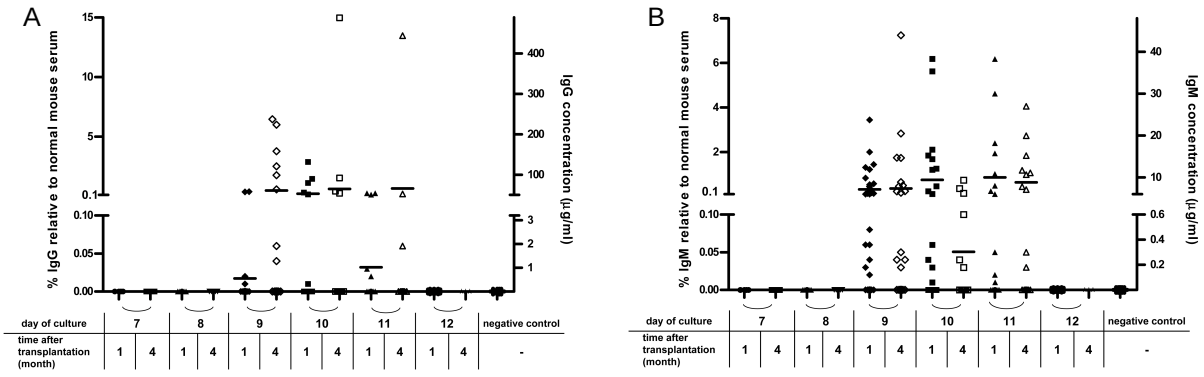

Figure 6-19: IgM (A) and IgG (B) levels in sera of Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice transplanted with differentiated ES cells. The percentages are relative to IgM (600µg/ml) and IgG (3200µg/ml) levels in serum of normal C57BL/6 mice. Closed symbols - serum Ig after 1 month; open symbols - serum Ig after 4 months.

6.3.3 ES cell-derived progenitors reconstitute B cells in Rag2$^{-/-}$ $\gamma_c^{-/-}$ mice for 4 months

To investigate the existence of Ig-producing cells lymphoid organs of the mice transplanted with ES cells differentiated *in vitro* for 9, 10 or 11 days were assayed by FACS for the presence of B cells 4 months after transplantation, i.e. for a long-term B cell reconstitution potential of the transplanted cells. In order to detect donor-derived B lineage cells the H2K^{b+} cells were further analysed for expression of B220, CD19 and surface IgM (Figure 6-20A).

No H2K^{b+} cells were found in mice transplanted with cells from day 7, 8 and 12 of *in vitro* ES cell differentiation. Likewise, no H2K^{b+} cells could be detected in any mice that had undetectable levels of IgM in their serum (Table 6-1). Combining all experiments only 5 of the 16 serum IgM$^+$ mice transplanted with ES cells differentiated *in vitro* for 9 days had detectable H2K^{b+}CD19$^+$ and IgM$^+$ bone marrow cells. Two out of 7 mice transplanted with 10 days-differentiated cells, and 4 out of 5 transplanted with 11 days-differentiated cells had detectable CD19$^+$ and IgM$^+$ cells in their bone marrow (Figure 6-20B, C left). Most CD19$^+$ cells in bone marrow were IgM$^+$), but IgD-negative, indicating that immature B cells, but no detectable numbers of earlier stages of B-lineage cells were present (Figure 6-20A top).

In the spleen H2K^{b+}CD19$^+$ and IgM$^+$ B cells were found in almost all serum IgM-positive mice transplanted with day 9, 10 and 11 ES cell-differentiated cells. Again, as in the case of serum levels, absolute numbers of donor-derived B cells originating from differentiated ES cells in the spleen only reached 0.05-0.5% of those developed in control transplantations with mouse bone marrow (Figure 6-20B, C middle). Highest absolute numbers were found in mice transplanted with day 9 cells, and they appear to correlate with the higher numbers of donor-derived B cells found in the bone marrow. In most recipients almost all of the mature B cells

(IgM^+ $CD19^+$ $CD93^-$) were $CD21^+$ $CD23^+$, a phenotype associated with follicular B cells (Figure 6-20A middle).[200]

ES cell-derived $H2K^{b+}CD19^+$ and IgM^+ cells were also found in the peritoneal cavity, again at days 9 and 10 of *in vitro* ES cell differentiation with the highest numbers (Figure 6-20B, C right). In most cases around 50% of the $CD19^+$ cells found in the peritoneal cavity showed a $CD5^+$ B1a phenotype (Figure 6-20A bottom).

The FACS analyses of the thymus of transplanted mice for lymphoid cells were difficult and only in a few cases $H2K^{b+}CD4^+$ donor-derived cells were found in the spleen. This might be so because the thymus of $Rag2^{-/-}$ $\gamma_C^{-/-}$ recipients is severely underdeveloped, and not receptive for a suboptimal number of progenitor cells.

In a second set of transplantations GFP-expressing GK3 ES cells were transplanted into sublethally irradiated $Rag2^{-/-}$ $\gamma_C^{-/-}$ C57BL/6 mice. The reconstitution efficiency of B cells in bone marrow, spleen and peritoneal cavity showed similar results. Again, no cells could be found in the thymic rudiments.

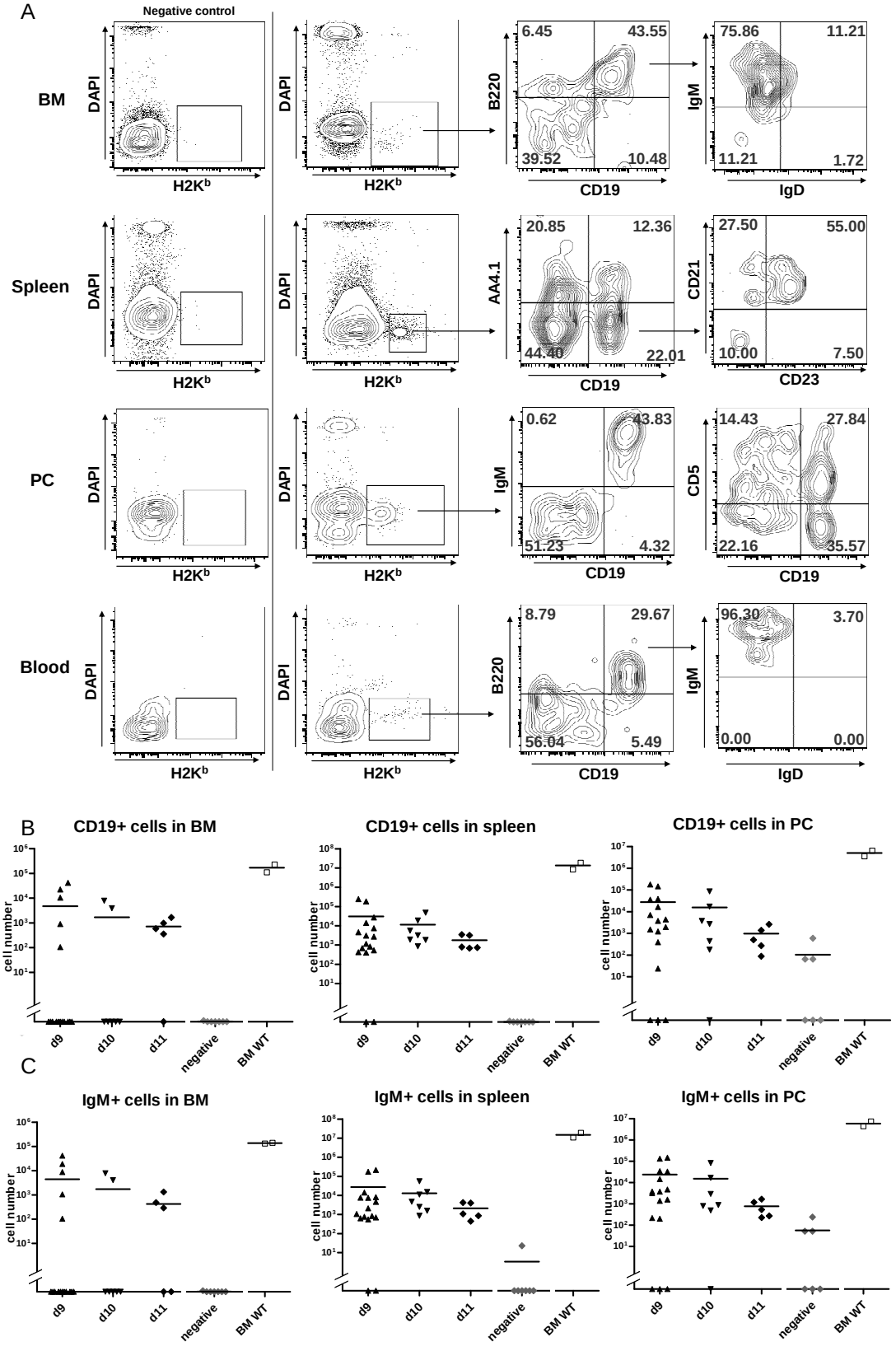

Figure 6-20: B cells in the bone marrow (BM), spleen, peritoneal cavity (PC) and blood 4 months after transplantation of Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice with non-transduced differentiated ES cells.
(A) Surface staining of BM , spleen, PCl and blood cells from Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice transplanted with ES cells cultured *in vitro* for the indicated time (H2K^b haplotype). Representative FACS stainings for each organ are shown. Cells are gated on living cells. Negative control shows H2K^b staining from non-transplanted Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice. Absolute cell numbers of CD19 (B) and IgM (C) expressing ES cell-derived cells in the BM (one tibia and femur), spleen and PC (for cell recovery see Materials and Methods). Number of analysed serum IgM-positive mice: day 9 N=16; day 10 N=7; day 11 N=5.

In order to test the reproducibility of the *in vitro* differentiation protocol that uses FCS and the media conditioned by hybridoma supernatants secreting SCF or Flt-3L these *in vitro* ES cell differentiation and *in vivo* transplantation experiments were performed with two additional FCS batches, as well as in the presence of recombinant SCF and Flt-3L. The results of these experiments showed that the hematopoietic progenitors that develop from cells in media containing different FCS reconstitute with similar numbers of CD19 and IgM positive cells after transplantation into Rag2$^{-/-}$ $\gamma_c^{-/-}$ recipient mice one month after transplantation (Figure S2A and B). From the findings that the B cell reconstitution of immunodeficient mice is not changed one and 4 months after transplantation I would suggest that this is also the case for the other, suitable selected FCS batches.

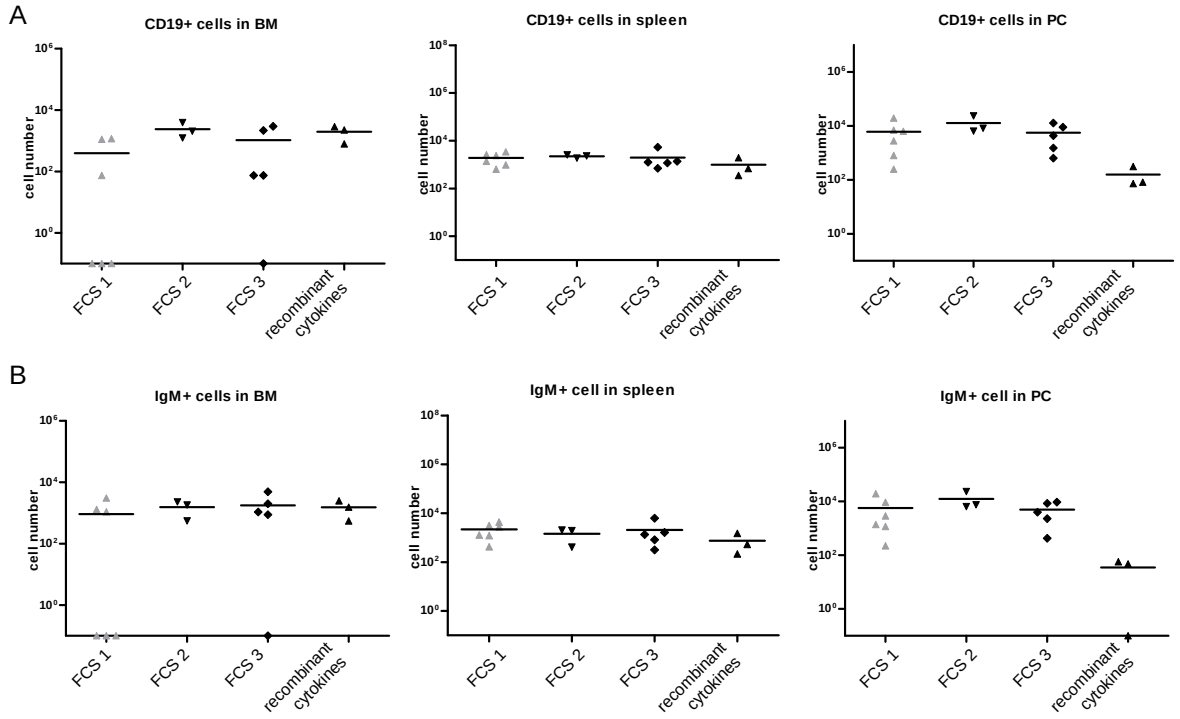

Figure 6-21: B cells in the bone marrow (BM), spleen and peritoneal cavity (PC) of Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice transplanted with *in vitro* differentiated ES cells in cultures (day 10) containing different batches of FCS or recombinant cytokines. Absolute cell numbers of CD19 (A) and IgM (B) expressing ES cell-derived cells in the bone marrow BM, spleen and PC determined by FACS.

6.3.4 ES cell-derived progenitors reconstitute myeloid cells in Rag2$^{-/-}$ $\gamma_c^{-/-}$ mice for 4 months.

Donor-derived cells from the spleen and peritoneal cavity of transplanted mice were analysed for expression of the myeloid markers Gr1 and Mac1 (CD11b) 4 months after transplantation. In the peritoneal cavity around 50% of the H2K^{b+} cells were Mac1^{+}, 15% of those also

expressed Gr1 (Figure 6-22A). The absolute numbers of $Mac1^+$ cells found in the bone marrow, spleen and peritoneal cavity of mice transplanted with cells from ES cells differentiated *in vitro* for 9, 10 or 11 days were small, but significantly above background levels. Compared to mice transplanted with C57BL/6 wild-type bone marrow cells the numbers are 1000 to 5000 times lower (Figure 6-22B). The myeloid compartment of the spleen and peritoneal cavity of $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice was most effectively reconstituted with ES cells transplanted after 9 days of *in vitro* differentiation.

From these experiments it can be concluded that a low number of transplantable hematopoietic progenitors with B lymphoid and myeloid, long-term (i.e. 4 months) repopulating potential develop when ES cells are induced *in vitro* by a protocol that allows the consecutive development of $Flk\text{-}1^+$, then $CD41^+$ and finally $CD45^+$ cells. This development appears to occur in a wave, detectable at days 9, 10 and 11 of ES cell differentiation, but not before or after.

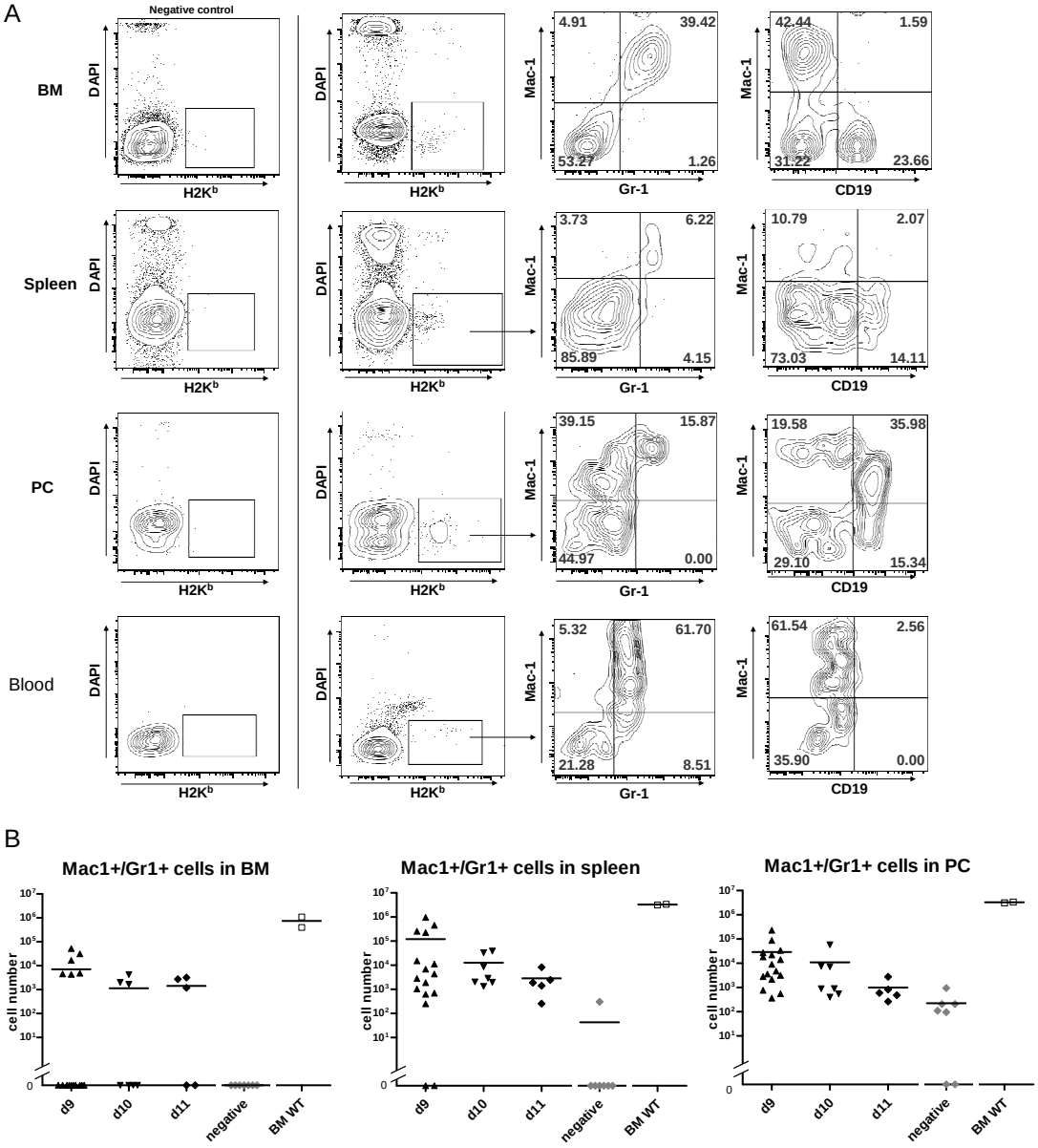

Figure 6-22: Myeloid cells in the bone marrow (BM), spleen, peritoneal cavity (PC) and blood 4 months after transplantation of Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice with differentiated ES cells. (A) Surface staining of cells from Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice transplanted with ES cells (H2K^b haplotype) cultured for the indicated time. Representative FACS stainings are shown. Cells are gated on living cells. Negative control shows H2K^b staining from non-transplanted Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice. (B) Absolute cell numbers of Mac1/Gr1 expressing ES cell derived cells in the BM (one tibia and femur), spleen and PC (for cell recovery see Materials and Methods). Number of analysed serum IgM-positive mice: day 9 N=16; day 10 N=7; day 11 N=5.

Again, the same myeloid reconstitution was achieved when the two additional FCS batches or recombinant preparations of SCF and Flt3-L were used to develop the hematopoietic progenitors in the culture, follwed by transplantation (Figure 6-23).

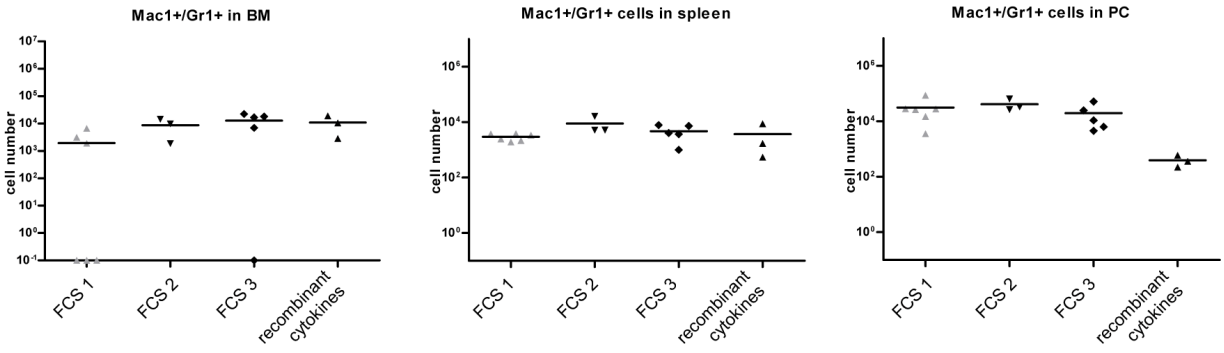

Figure 6-23: Myeloid cells in the bone marrow (BM), spleen and peritoneal cavity (PC) of Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice transplanted with *in vitro* differentiated ES cells in cultures (day 10) containing different batches of FCS or recombinant cytokines. Absolute cell numbers of Mac1/Gr1 expressing ES cell-derived cells in the bone marrow, spleen and peritoneal cavity determined by FACS.

6.3.5 HOXB4 transduction enhances the repopulating ability of ES cell-derived progenitors

Ectopic, constitutive HOXB4 expression in HSCs has been found to allow their *in vitro* expansion for prolonged periods of tissue culture without loss of long-term hematopoietic repopulation potential upon transplantation. A HOXB4-expressing, puromycin-selectable retroviral vector has been shown to transfect HSCs and to allow their proliferative expansion *in vitro* [194].

Since the experiments shown above suggested that only a small number of hematopoietic progenitors could develop in the *in vitro* differentiating ES cell cultures I attempted to find optimal conditions, and to determine the optimal time of *in vitro* differentiation to expand these cells by HOXB4 transduction. GK3 as well as J1 ES cells were induced to differentiate *in vitro* for 5 to 12 days and then transduced with the HOXB4-puromycin resistance vector. The transduced cells were selected in puromycin-containing HSC-growth medium, i.e. in the presence of SCF, TPO, IL-3 and IL-6 on irradiated, semi-confluent OP9 stromal cells [201]. Cell lines proliferating for approximately two weeks could be established with high efficiencies from day 5 to day 12-differentiating ES cell cultures. $5x10^6$ cells were transplanted into Rag2$^{-/-}$ $\gamma_C^{-/-}$ C57BL/6 or Rag2$^{-/-}$ $\gamma_C^{-/-}$ Balb/c recipient mice, respectively. Bone marrow cells of wild-type C57BL/6 mice prepared *ex vivo*, not transduced by HOXB4, served as positive controls after transplantation into the same recipients.

Cells transduced between day 5 and 10 in the culture reconstituted the B cell compartment, so that IgM and IgG were detectable in the sera of Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice (Table 6-2). About half of the mice transplanted with HOXB4-tranfected cells contained IgM and IgG, indicating that the transplanted progenitors, transduced and expanded by HOXB4, remained limited in

numbers comparable to numbers of progenitors of non-HOXB4-transduced progenitors transplanted at day 9 to 11. (Table 6-1, Table 6-2). None of the mice transplanted with differentiating ES cells transduced with HOXB4 at day 11 or 12 did yield serum IgM and IgG in their blood (Table 6-2).

In conclusion these experiments show that *in vitro* differentiating ES cells can be stably transduced with HOXB4 as early as day 5, but no later than day 10 to yield progenitors with the capacity to reconstitute myeloid and lymphoid cell lineages in the transplanted $Rag2^{-/-}$ $\gamma_C^{-/-}$ host.

	Days of *in vitro* differentiation and HOXB4 transfection			
	5/6	**7/8**	**9/10**	**11/12**
	Number of mice with Ig in the serum **/** Total number of transplanted mice			
IgM	15/22 (68%)	9/18 (50%)	8/16 (50%)	0/10
IgG	15/22 (68%)	9/18 (50%)	8/16 (50%)	0/10

Table 6-2: Frequencies of mice receiving transplants of HOXB4-transduced differentiating ES cells with detectable Ig in their sera. ES cells transdued with HOXB4 at indicated times of culture with subsequent expansion for approximately two weeks (see Materials and Methods).

All serum Ig-positive mice were analysed by FACS 4 months after transplantation for the presence of donor (ES cell-) derived cells in BM, thymus, spleen, blood and peritoneum. The GFP-expressing (for the case of ES cell-derived transplants) or $H2K^{b+}$ (for the case of wild-type bone marrow transplants) cells were further characterised for B-, T- and/or myeloid lineage cells. Donor-derived lymphocytes could be found in bone marrow, spleen, blood, peritoneal cavity and in some cases even the thymus. The numbers of B-lymphoid and myeloid cells found in mice transplanted with cells transduced on day 5 or 6, day 7 or 8 and day 9 or 10 are similar (Figure 6-24), but all are approximately 5 to 10-fold higher than those found in mice transplanted with non-transduced cells transplanted between day 9 and 11. This could suggest that the *in vivo* stability of HOXB4-transduced progenitors with multilineage repopulation capacities might be increased, although their numbers are not significantly increased during our time of culture (compare Figure 6-20 and Figure 6-22 with Figure 6-24).

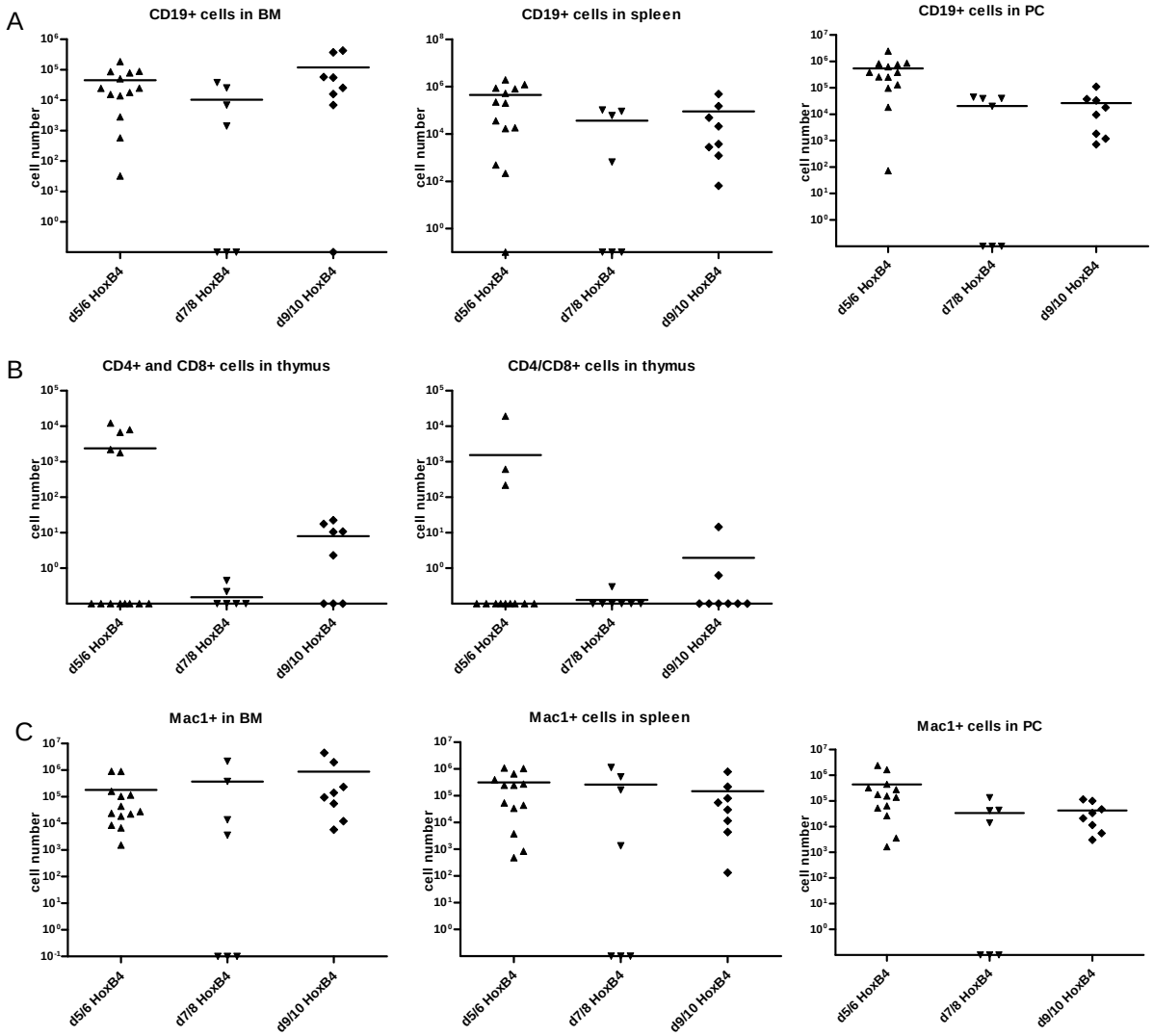

Figure 6-24: **Lymphoid and myeloid cells in the bone marrow (BM), spleen and peritoneal cavity (PC) 4 months in Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice transplanted with HOXB4-transfected differentiated ES cells.** Absolute numbers of cells of (A) CD19$^+$, (B) IgM$^+$ and (C) Mac1$^+$ cells in the indicated organs. ES cells transduced with HOXB4 at indicated time points and then expanded for two weeks in media containing IL-3, IL-6, SCF and TPO were transplanted into Rag2$^{-/-}$ $\gamma_C^{-/-}$.

The HOXB4-transduced, transplanted, GFP$^+$ cells derived from differentiated ES cells were found in the bone marrow at 4 months after transplantation (Figure 6-25A). This indicates that some of the transplanted cells were capable of homing to the bone marrow, and to populate it with progenitors, myeloid cells and precursor and immature B cells for 4 months, i.e. long-term, comparable to the control mice transplanted with normal bone marrow cells (Figure 6-25B). More specifically the patterns of B220/CD19 and IgM/CD19 expressing cells were comparable, although the percentage of B220$^+$CD19$^+$ and IgM$^+$CD19$^+$ cells were lower in bone marrow of mice transplanted with the HOXB4-transduced differentiated ES cells. Around 80% of the GFP$^+$ cells (70% of the cells from normal bone marrow as control) did not

express B-lineage-related markers, i.e. were $B220^{-}CD19^{-}IgM^{-}$ Around 12% of the HOXB4-transduced, differentiated ES cell-derived cells (and 23% of normal bone marrow-derived cells) were $B220^{+}CD19^{+}$, half of which were $sIgM^{+}$, the other half $sIgM^{-}$. Hence, the latter cells had the phenotype of preB cells. This pattern of B-lineage cells might be expected in bone marrow that is continuously generating B cell from progenitors and precursors. One third of the $CD19^{+}$cells also expressed Mac1, indicating that a more mature subset of B1 cells was detectable [202]. $CD19^{-}$ $Mac1^{+}$ $Gr1^{+}$ myeloid cells could not be found in the bone marrow.

In the spleen 60% of the GFP^{+} ES cell derived and 60% of the bone marrow derived cells were $CD19^{+}$ B cells. In contrast to mice transplanted with non-transduced differentiated ES cells, HOXB4-transduced cells showed a reconstitution of marginal zone ($CD21^{+}$ $CD23^{-}$), follicular ($CD21^{+}$ $CD23^{+}$) and transitional ($CD21^{-}$ $CD23^{low}$) B cells (Figure 6-25A). In the peritoneum half of the donor-derived cells in ES-cell transplanted mice and over 75% in the bone marrow transplanted mice were $CD19^{+}IgM^{+}$. In both cases two third of them were mature, $CD5^{+}$ B1a cells.

Interestingly, 10% of the $CD19^{+}$ cells in spleen of mice transplanted with HoxB4-transduced, day 5-differentiating ES cells were $CD93^{-}$ $sIgM^{-}$, i.e. had the phenotype of preB cells. This $CD19^{+}IgM^{-}$ population of B-lineage cells was also detected in peritoneum. In the control mice transplanted with normal bone marrow such cells were absent. I will discuss this difference below.

In conclusion these analyses of GFP^{+} B-lineage cells suggest that a long-term population of B-lineage cells in bone marrow has been established and, hence, that there appears to be continuous B cell development from progenitors and precursors established in the recipients by the transplantation of HOXB4-transduced cells of differentiated ES cells.

FACS analyses of CD4 and CD8-expressing, donor-derived GFP^{+} T-lineage cells in the thymus showed a population pattern that could be expected from a long-term establishment of progenitors and precursors in the thymus and a continuous generation of mature T cells in the spleen. In the thymus 60% of the GFP^{+} cells were $CD4^{+}CD8^{+}$double-positve cells, 18% $CD4^{+}$ single-positive, 9% $CD8^{+}$ single-positive and around 13% $CD4^{-}CD8^{-}$ double negative cells, a pattern expected from the population of the thymus and the spleen - with the characteristic ratio of 2:1 $CD4^{+}$ to $CD8^{+}$ cells, as seen in the recipients of control wild-type bone marrow cells (Figure 6-25A and B).

The numbers of donor-derived cells found in the mice that received HOXB4-transduced, day 5-differentiating ES cells were lower than those receiving control wild-type bone marrow cells (Figure 6-25C).

In contrast to non-transduced, differentiated ES cells, and in contrast to wild-type bone marrow transplants, $Gr1^+$ myeloid cells were undetectable, while $Mac1^+$ $Gr1^-$ cells appeared in spleen and peritoneum of mice transplanted with HOXB4-transduced, differentiated ES cells.

Altogether these results suggest that ES cell lines, other than those used in this study, are capable of generating lines of HOXB4-transduced hematopoietic progenitors that are able to long-term reconstitute lymphoid and myeloid compartments of severe combined immunodeficient $Rag2^{-/-}$ $\gamma_C^{-/-}$ mice.

The quantities of donor-derived lymphoid and myeloid cell populations in BM, thymus spleen and PC, determined at 4 months after transplantation of day 9-differentiated ES cells, of differentiated, HOXB4-transduced ES cells, and of normal bone marrow cells as controls (Figure 6-25C) can be used as measures to compare their effectiveness of long-term repopulation. Normal bone marrow, clearly, has the best repopulation capacity. The HOXB4-transduced, ES cell-derived cells showed approximately half of that potency, visible in the quantities of donor-derived cells in all organs.

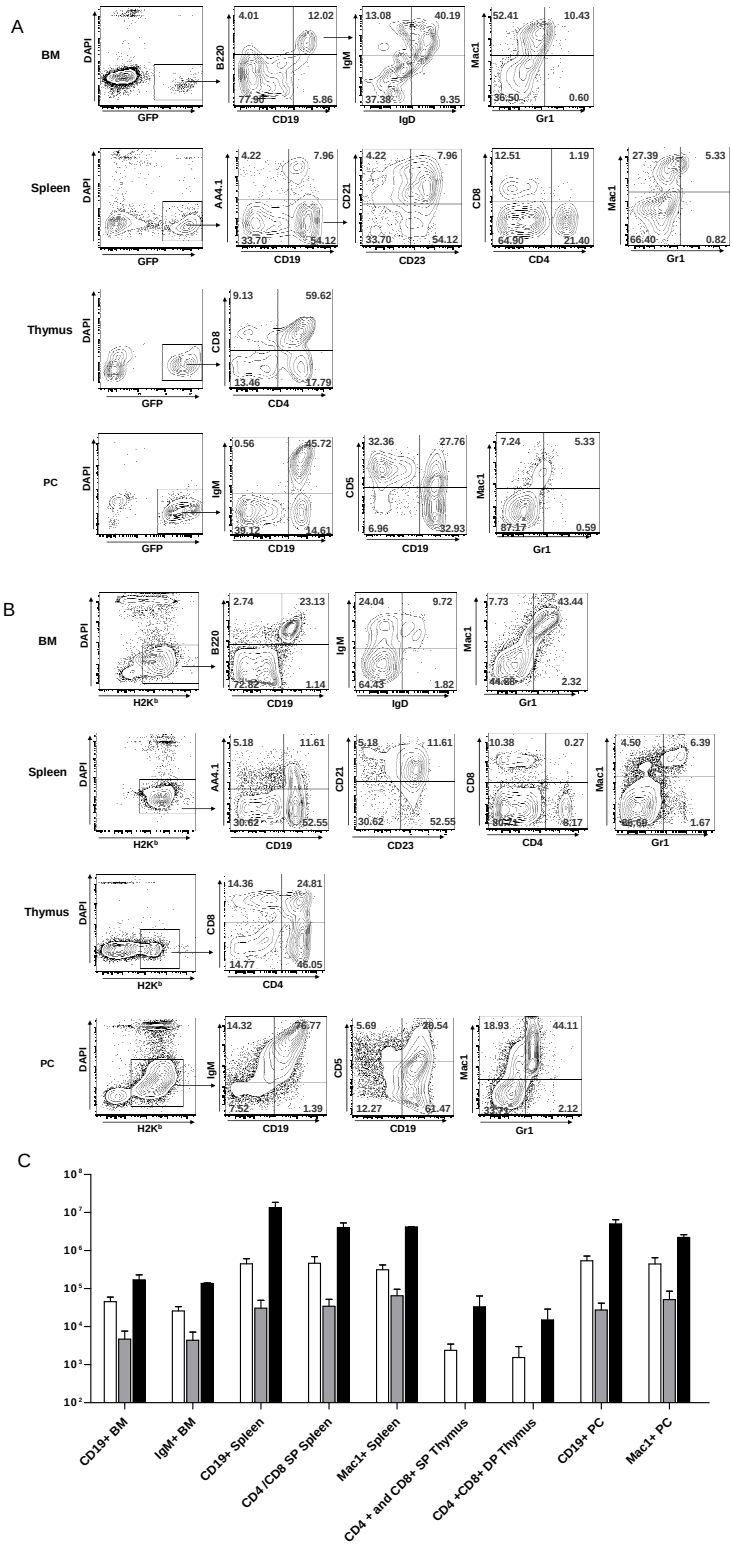

Figure 6-25: HoxB4 transduced differentiated ES cells reconstitute the B, T and myeloid compartment of Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice. Surface staining of bone marrow (BM), spleen, thymus and peritoneal (PC) cells of mice transplanted with HOXB4-transduced GK3 ES cells (GFP$^+$) (day 5 or 6) (A) and wild-type BM (B) for expression of B, T and myeloid markers. One representative FACS staining of the analysed cells for every organ is shown. In (C) absolute cell numbers are shown of B cells in the BM; B, T and myeloid cells in the spleen; T cells in the thymus, B and myeloid cells in the PC derived from J1 ES cells that were transduced on day 5 or 6 (white bars) compared to non-transduced ES cells from day 9 of the culture (grey bars), and to wild-type BM (black bars) in transplanted Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice. SP: CD4$^+$ or CD8$^+$ single positive T cells, DP: CD4$^+$ CD8$^+$ double positive T cells.

6.4 Establishment of a reporter system to detect lymphoid progenitors at their point to become either T- or B- lymphoid committed

6.4.1 Cloning preTα and λ5 reporter BAC clones

BAC clones have the advantage to contain large parts of chromosomes, so that when transgenic reporter systems are generated they contain most of the regulatory regions of the gene of interest in the proper chromatine structures. To follow B and T cell development *in vitro* and *in vivo* BAC clones were chosen so that they contain large parts of chromosome 16 or 17 spanning λ5 or preTα centrally (BAC bMQ-282I11 for λ5, BAC bMQ-353M7 for preTα), respectively. The targeting cassette, carrying the fluorescence and resistance gene, was constructed following usual cloning strategies (PCR, restriction digest and ligation). Homologous oligonucleotides were chosen so that the 40nt are homologous to the targeting cassette and 40nt are homologous to the targeted BAC vector (Figure 6-26A and B)). The cassette was inserted using the ATG translation start from the targeted gene and a polyA signal was introduced to avoid the production of fusion products. To avoid uncontrollable splicing processes and loss of regulatory regions the original BAC was not deleted of any sequence at any point of the construction.

YFP was used as the reporter for preTα and GFP as the reporter for λ5. The BACs were constructed in bacteria, so that after homologous recombination several clones for each BAC were established. The clones were digested with NcoI and PstI and the gel electrophoresis pattern was compared to the digested unmodified BAC control for presence or absence of bands (Figure 6-26C and D).

Clones showing differences in the digestion pattern were sequenced 5’ and 3’ of the the insertion with the primers preTα 5’sequ and preTα 3’sequ or λ5 5’sequ and λ5 3’sequ, respectively. One correct clone for each modified BAC was used to establish transgenic ES cells and mice.

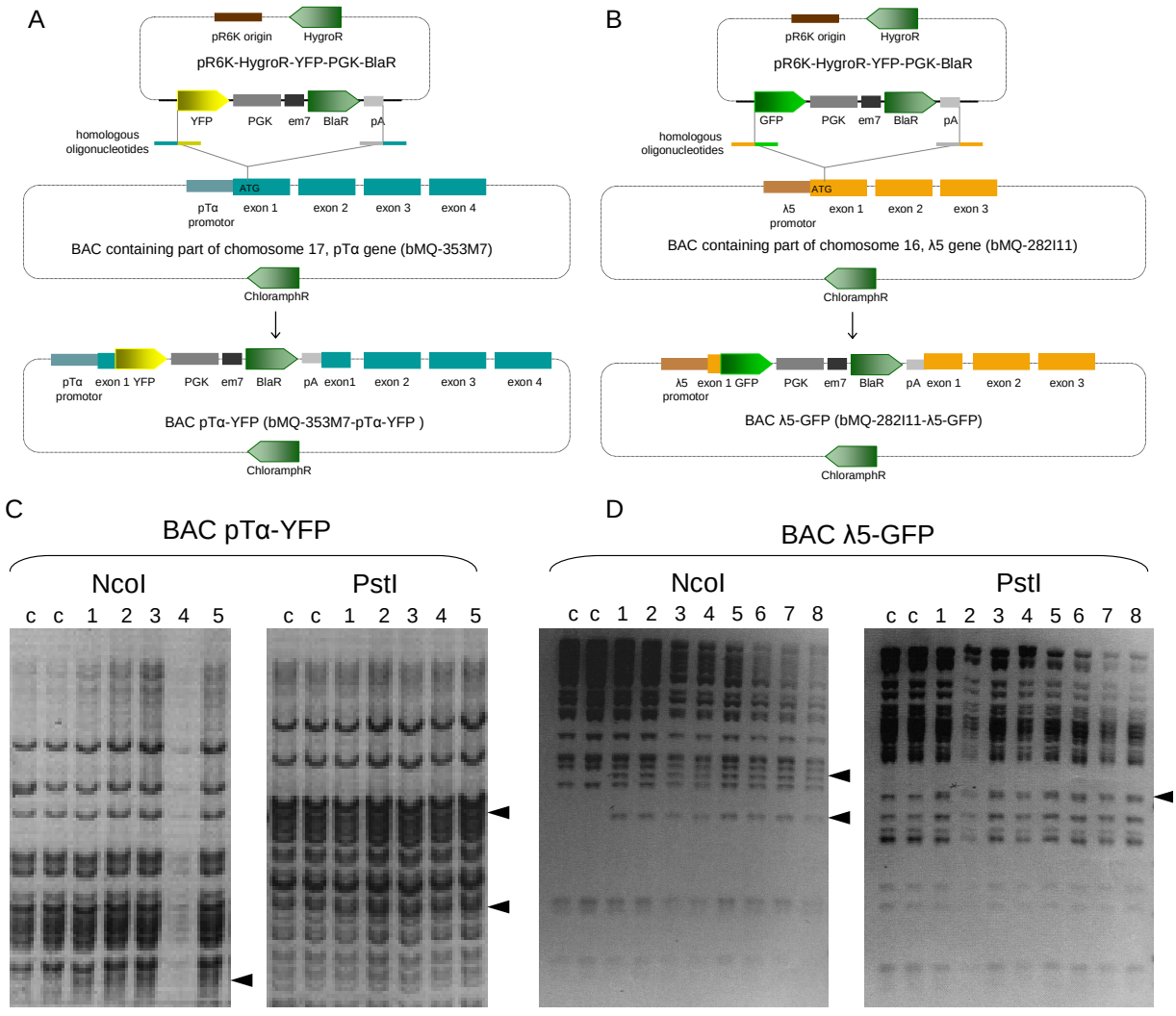

Figure 6-26: Cloning strategy for the construction of the preTα-YFP (A) and λ5-GFP (B) BAC. Modified BACs (1-7 or 1-8) were digested with NcoI and PstI and gel pattern was compared to non-modified controls (c) (C, D). Arrows indicate additional or missing bands.

6.4.2 Generation of ES cell lines carrying preTα and λ5 reporter BAC clones

Embryonic fibroblast (EF) -free J1 ES cells were stably transfected with the BAC preTα-YFP or the BAC λ5-GFP to generate BAC-transgenic ES cell lines. The cells carrying the BAC were selected with blasticidin and from $3x10^5$ cells approximately 10 clones grew out that were picked. Genomic DNA was isolated to reconfirm positive clones by PCR. Therefore the primers GFP/YFP rev and preTα 5'sequ or λ5 5'sequ, respectively, were used (Figure 6-27). After establishment of the ES cell clones they were further tested for alkaline phosphatase activity to ensure that they were still in an undifferentiated stage after the transfection and selection procedure (Figure 6-27).

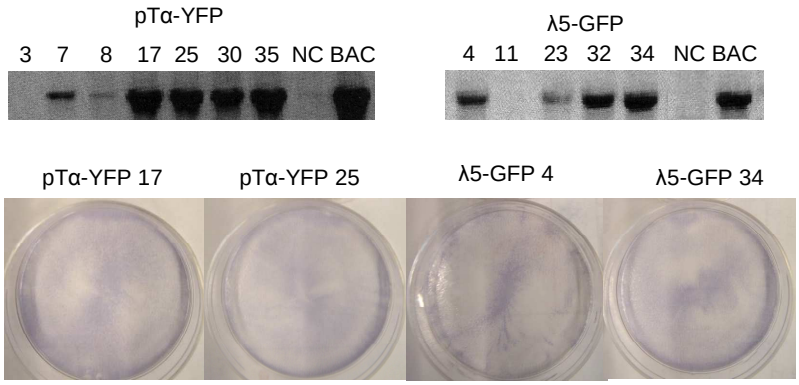

Figure 6-27: Confirmation of transgenic ES cell lines by PCR and pluripotency check by alkaline phosphatase test (alkaline phophatase-positive cells turned blue).

6.4.3 Reporter gene expression of BAC preTα-YFP ES cells upon induction of T cell differentiation in vitro

Expression of preTα has been seen to be T lymphocyte lineage specific, i.e. in thymic development it is expressed from the DN3 throughout the beginning of the DP stage of T cell development by RT-PCR (see 3.2.3) [133]. Therefore, BAC preTα-YFP ES cells were expected to become YFP^+ as soon as they differentiate and enter the DN3 stage of T cell development *in vitro*. J1 ES cells transgenic for BAC preTα-YFP were differentiated *in vitro* into B and T lineage cells to confirm proper expressing of the reporter genes under the respective conditions (Figure 6-1, Figure 6-5). Differentiation of the cells towards T lineage (Figure 6-5) resulted in expression of YFP. Cells in the very early stages of T cell development (DN1 and DN2) did not express YFP. From the DN3 stage on they became YFP^+, so that DN3, DN4, DP and SP cells in the culture were YFP^+ (Figure 6-28B). (For characterisation of the developmental stages of T cell development see 3.2.2). In conclusion YFP was switched on at the expected stage of development, but the cells stayed YFP^+ even after reaching the DP stage *in vitro*.

Following the protocol to induce preB cell development (Figure 6-1) the cells developed into $B220^+$ $CD19^+$ cells but did not express YFP (Figure 6-28A). This result confirms that preTα is expressed during early T cell development but not during B cell development.

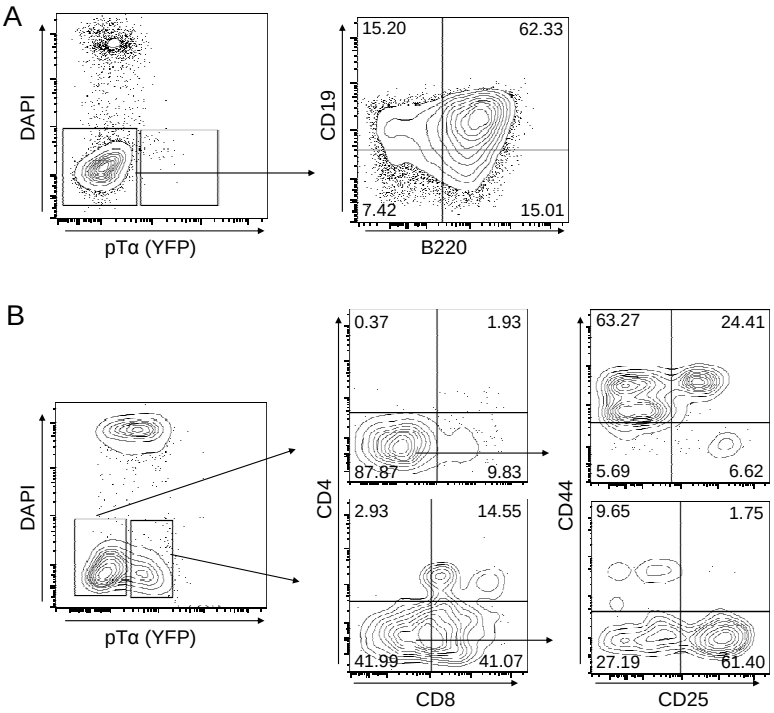

Figure 6-28: FACS anaylsis on day 19 of *in vitro* differentiation of BAC preTα-YFP transgenic J1 ES cells under B cell (A) and T cell (B) conditions. Numbers in quadrants represent percentages of living lymphocytes.

6.4.4 Reporter gene expression of BAC λ5-GFP ES cells upon induction of B cell differentiation in vitro

Cells are expected to express λ5 as they differentiate into B lineage cells starting at the proB cell stage and downregulate it at small preB II and immature B cell stage. To test this J1 ES were differentiated into B and T lineage cells *in vitro* (Figure 6-1, Figure 6-5).

The cells expressed GFP only if they were induced to become B lineage cells. 95% the GFP^+ cells express B220 and/or CD19 only 40% of the GFP^- cells (Figure 6-29A). None of the cells expressing T lineage specific surface markers after differentiation under T cell conditions became GFP^+ (Figure 6-29B).

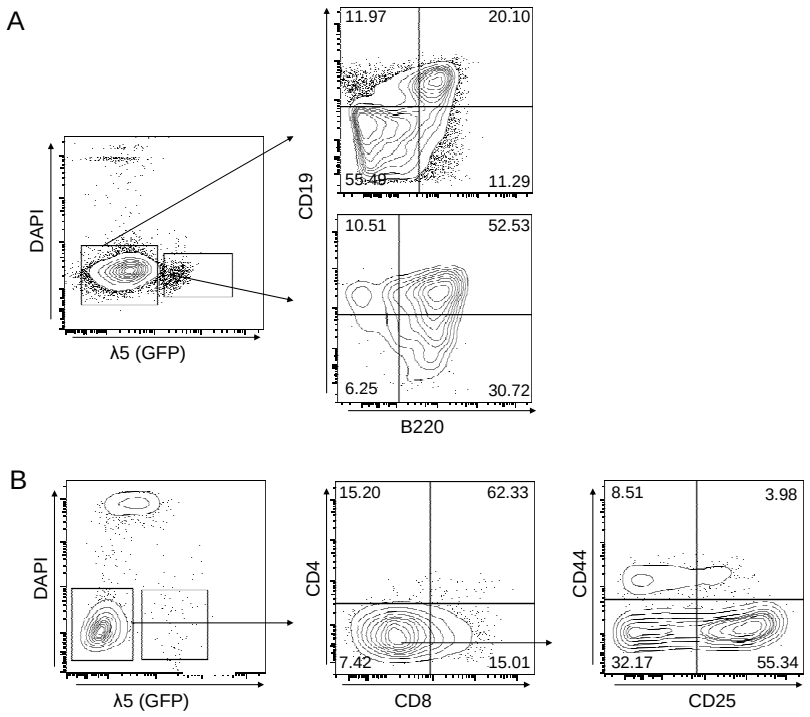

Figure 6-29: FACS anaylsis on day 19 of *in vitro* differentiation of BAC λ5-GFP transgenic J1 ES cells under B cell (A) and T cell (B) conditions. Numbers in quadrants represent percentages of living lymphocytes.

6.4.5 Generation of transgenic preTα and λ5 reporter mice

In separate experiments the BAC preTα-YFP and the BAC λ5-GFP were injected into the pronucleus of C57Bl/6 oocytes and transferred into pseudo-pregnant female C57Bl/6 mice by Dr. Uwe Klemm and Karin Bordasch at the Max Planck Institute for Infection Biology, Berlin. Littermates were analysed by PCR using the primers GFP/YFP rev and preTα 5'sequ or λ5 5'sequ (see 5.1.8), respectively. Positive mice were always bred with C57Bl/6 to ensure that all littermates are heterozygous, i.e. comparable, because the PCR cannot distinguish between heterozygous and homozygous mice.

So far, only BAC preTα-YFP mice are available. The injection of BAC λ5-GFP was not yet successful.

After establishment of the transgenic BAC preTα-YFP mouse strain, it was crossed with the established transgenic λ5-huCD25 mice[129, 130] to yield preTα /λ5 double transgenic reporter mice (Figure 6-30).

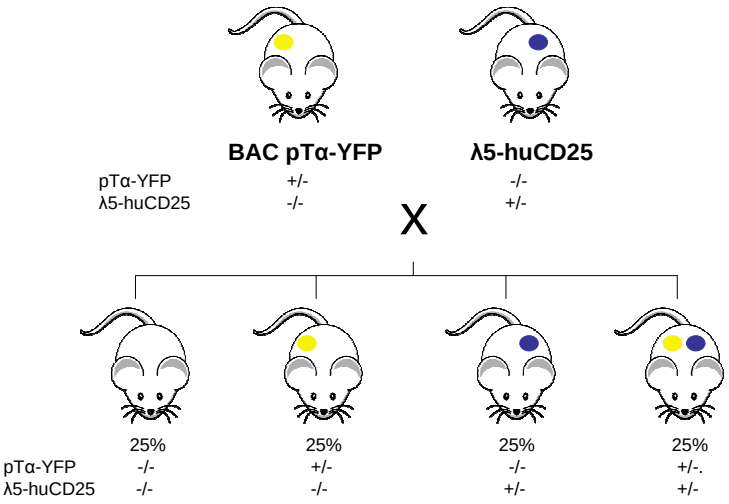

Figure 6-30: Breeding scheme for the generation of BAC preTα-YFP/λ5-huCD25 double transgenic reporter mice.

6.4.6 Analysis of BAC preTα-YFP/λ5-huCD25 double transgenic reporter mice

Early development of B cells occurs in the bone marrow, and T cells mature in the thymus. Since CLPs have been found in the bone marrow, preTα- and/or λ5-expressing cells were expected to be found in the bone marrow and the thymus. Therefore, these primary lymphoid organs were analysed for the presence of YFP^+ and/or $huCD25^+$ cells (Figure 6-31).

Approximately 15% of all bone marrow cells were found to be $huCD25^+$, 2.5% were YFP^+, and 0.1% were positive for both markers. In the thymus most cells were YFP^+. Among the YFP^+ cells two populations were identified, one being highly positive and one being positive to a lower extent for YFP. Only few cells that were $huCD25^+/YFP^-$ were found (<0.1%).

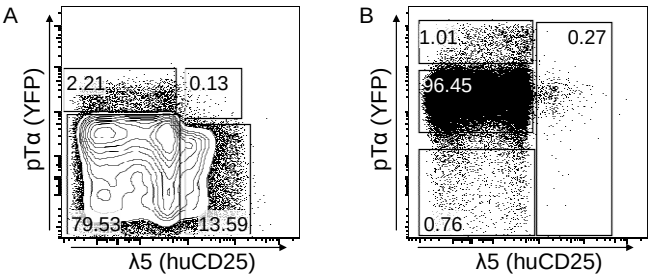

Figure 6-31: Analysis of bone marrow (A) and thymus (B) of preTα-YFP/λ5-huCD25 double transgenic mice for appearance of cells expressing one or both reporter genes. Numbers in quadrants are percentages of cells gated on living cells.

To determine the developmental stages in which the reporter genes are expressed known markers characterising hematopoietic, lymphoid, B and T lineage cells were used in combination with the reporter gene expression (Figure 6-32, see 3.2.2).

In the bone marrow LSK (lin^- $ckit^+$ $Sca\text{-}1^+$) cells including HSCs, MPPs and LMPPs did not express huCD25 nor YFP. A small fraction of cells expressing low levels of YFP could be detected within the CLP population (lin^- $ckit^{low}$ $Sca\text{-}1^{low}$ $IL\text{-}7R^+$) and around one third of the

proB cells ($B220^+$ $CD19^-$) were determined to express YFP (Figure 6-32B). Analysing all YFP^+ cells in the bone marrow for marker gene expression resulted in two phenotypically distinct populations - a $B220^+$ $ckit^-$ and a $B220^-$ $ckit^{low}$ population (Figure 6-32C), phenotypes which characterise preB cells and CLPs, respectively. 85% of the YFP^+ cells in the bone marrow have the $B220^+$ $ckit^-$ preB cell-like phenotype, whereas 11% were $B220^-$ $ckit^+$ CLP cell-like.

Half of the cells in the preB I and one third of the cells in the preB II cell stage expressed huCD25 but not YFP (Figure 6-32B and C). From the immature B cell stage on only very few cells expressed huCD25. Among the $huCD25^+$ cells in the bone marrow over 90% had a preB I or preB II phenotype characterised by surface expression of B220 and CD19 and the absence of sIgM-expression. Again, only very few cells were in the proB or immature and mature B cell stage.

The few double positive cells (YFP^+ $huCD25^+$) were found in the proB cell population ($B220^+$ $CD19^-$) (Figure 6-32C).

In conclusion the phenotypes of cells found in the present study, i.e. $huCD25^+$ and/or YFP^+ resemble those of previous studies where CLP have been found in the bone marrow expressing preTα and λ5 in the same cell[136] (see 3.2.3).

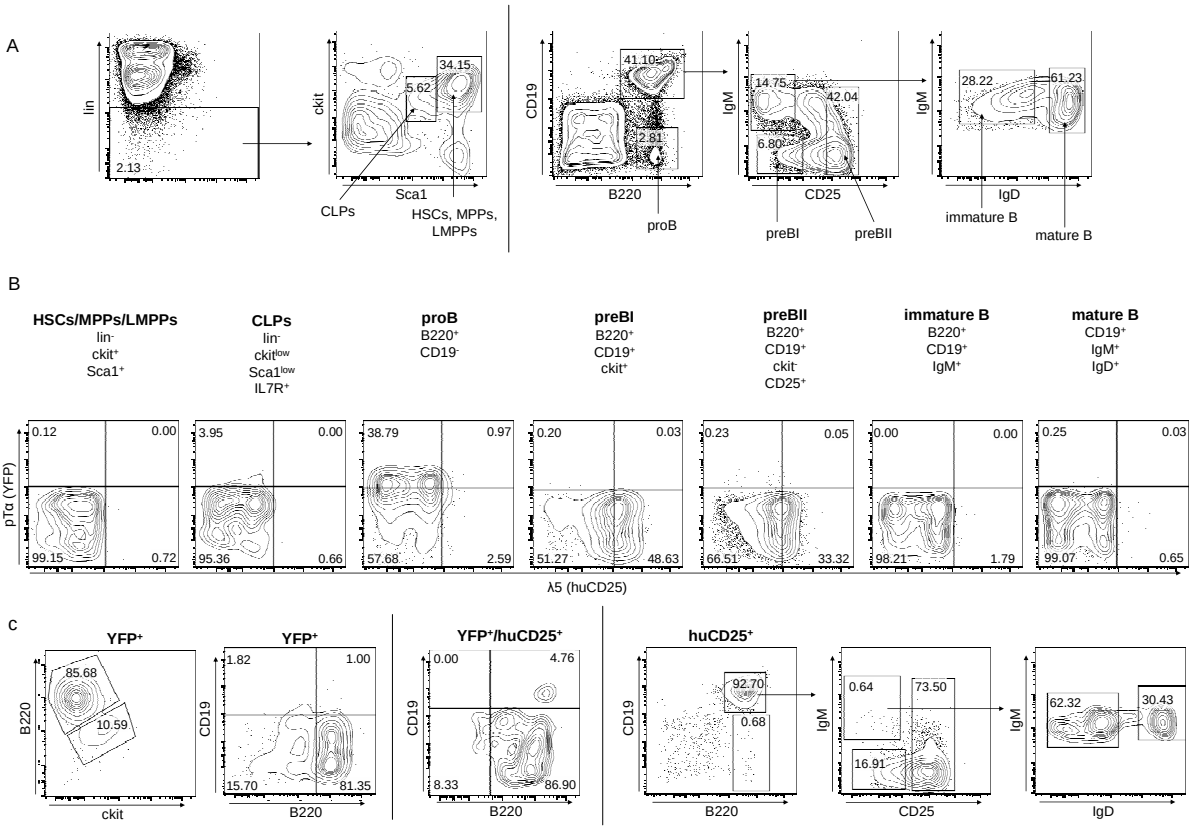

Figure 6-32: Characterisation of different hematopoietic stages (A) in the bone marrow of preTα-YFP/λ5-huCD25 double transgenic mice for expression of YFP and huCD25 (B). Cells are first gated on cells expressing the reporter genes followed by characterisation of different stages using known surface markers (C). Dead cells are discriminated by DAPI staining. lin: B220, CD3, CD4, CD8, Ter119, Mac-1, Gr-1, CD11c, NK1.1

The reporter gene-positive cells in the thymus were analysed using surface markers defining the different stages of T cell development (Figure 6-33A, see 3.2.2). One third of the cells in the DN1 stage, over 90% of the cells in the DN2 and DN3 stage and more than 80% of the cells in the DN4 stage were YFP^+. None of them were $huCD25^+$.

The level of YFP protein differed between the different proT cell population (Figure 6-33B). Thus, the cells in the DN3 and some of the DN2 cells were positive for YFP to a higher extent than in all other stages.

Almost all cells in the DP stage were YFP^+, with <0.5% also $huCD25^+$. In the SP stage around half of the cells were YFP^+.

The opposite analysis was performed to define the frequencies of cells with a given phenotype among the reporter gene expressing cells. The YFP^+ cells in the thymus could be subdivided into two populations, a YFP^{int} and a YFP^{high} population. 80% of the YFP^{high} cells are DN3 and 15% DN2 proT cells. YFP^{int} cells are found within all developing T cell populations, whereas YFP^+ $huCD25^+$ are only found in the DP cell population.

In contrast to expression of the YFP from the transgenic preTα locus normal expression of preTα is downregulated at the DP T cell stage which might be a result of a long half-life of the YFP or an improper downregulation of the transgene.

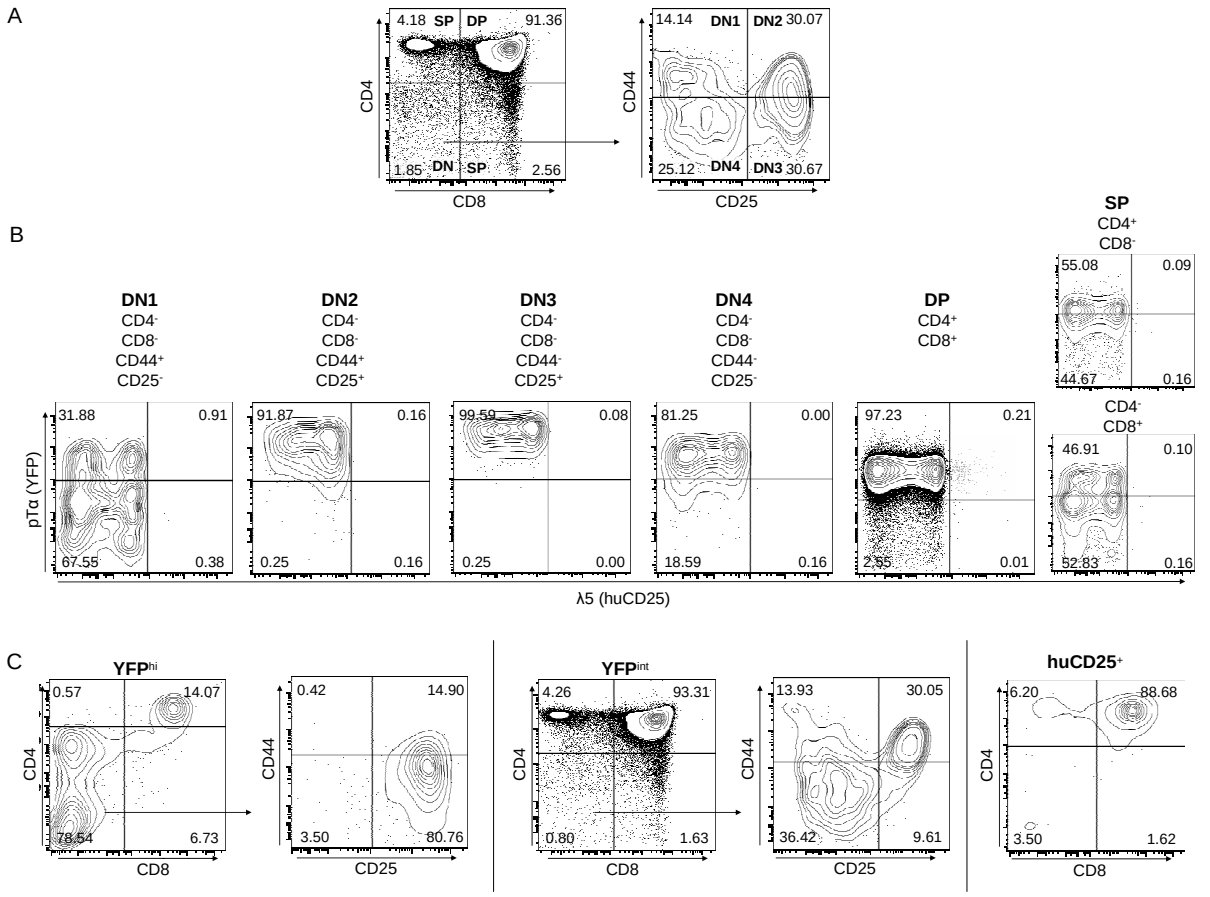

Figure 6-33: Characterisation of different T cell developmental stages (A) in the thymus of preTα-YFP/λ5-huCD25 double transgenic mice for expression of YFP and huCD25 (B). Cells are first gated on cells expressing the reporter genes followed by characterisation of different stages using known surface markers (C). Dead cells are discriminated by DAPI staining.

6.4.7 Analysis of the in vitro developmental potential of YFP$^+$ and/or huCD25$^+$ cells

To evaluate whether the reporter gene expressing populations in the BM and the thymus have the potential to develop into B and/or T cells they were FACS sorted and cultured under conditions that favour the development of B or T cells (OP9 or OP9-DL1 stromal cells with IL-7 and Flt-3L, respectively). In order to investigate the hematopoietic potential of the reporter gene-positive cells on a single cell level, cells were FACS-sorted and cultured as single cells. The result would indicate how many cells of the population make B and/or T lineage cells. At the same time, 10 cell/well were also cultivated and analysed. This takes advantage of a possible community effect of the cells, but it remains unclear how many of the

cells in the culture started growing. For these experiments the cells of 3-4 mice had to be pooled to get sufficient numbers of cells to be sorted as indicated in Figure 6-31. The cells were cultured for 10 days on OP9 or OP9-DL1 stromal cells. Wells with detectable colonies were counted (Table 6-3) and FACS-analysed (Figure 6-34).

The huCD25$^+$/YFP$^-$ population from the BM contained the highest number of cells with the potential to grow under "B cell conditions" (OP9/IL-7/Flt-3L), but not under "T cell conditions" (OP9-DL1/IL-7/Flt-3L). The huCD25$^+$ cells of the thymus behaved similarly under "B cell conditions", but the phenotypes of the cells derived from those huCD25$^+$ populations were different. Whereas the huCD25$^+$ cells from the BM showed a huCD25$^+$ B220$^+$ YFP$^-$ expression pattern (Figure 6-34b), the huCD25$^+$ cells from the thymus did neither express huCD25 nor B220 after cultivation, but some cells remained YFP$^+$ upon cultivation (Figure 6-34d). Under "T cell conditions" some of the huCD25$^+$ cells of the thymus grew and developed into YFP$^+$ cells and lost huCD25 expression (Figure 6-34g).

YFP^{high} huCD25$^-$ cells from the thymus (Figure 6-34h) as well as YFP$^+$ huCD25$^-$ from the BM (Figure 6-34e) had only the potential to grow under "T cell conditions". In contrast to cells from the BM cells from the thymus grew in higher frequencies. In both cases the grown cells were YFP$^+$ (Figure 6-34e and h). YFP^{int} could not develop colonies *in vitro* under "B and T cell conditions".

Double-positive (YFP$^+$/huCD25$^+$) cells that could be sorted from the BM did not proliferate either on OP9 or on OP9-DL1 stromal cells, i.e. under "B or T cell conditions". The cells could not be sorted out from the thymus because of very low numbers and are therefore among the huCD25$^+$ cells.

YFP$^-$ huCD25$^-$ cells from BM could proliferate under "B cell conditions", but not under "T cell conditions", while the few YFP$^-$ huCD25$^-$ cells from the thymus grew under both conditions. Interestingly, the colonies developed on OP9 stromal cells did not express any of the two reporter genes but were Mac-1$^+$ (Figure 6-34a). By contrast, the double-negative thymocytes became YFP$^+$ under "T cell conditions" (Figure 6-34f).

In conclusion, huCD25$^+$ cells from the BM as well as from the thymus could only proliferate under "B cell conditions" while YFP$^+$ cells from BM and YFP^{high} cells from the thymus could only proliferate under "T cell conditions". Double-positive as well as double-negative cells from the BM had no or only low potential to proliferate in *in vitro* coculture with OP9 ("B cell conditions") or OP9-DL1 stromal cells ("T cell conditions").

sorted cell population	condition for cultivation of sorted cells					
	OP9 Flt-3L/IL-7			**OP9-DL1 Flt-3L/IL-7**		
	number of wells with growing cells (10days after sort and seed)		phentotype after cultivation Figure 6-34	number of wells with growing cells (10days after sort and seed)		phenotype after cultivation Figure 6-34
	1	**10**		**1**	**10**	
BM huCD25$^-$/YFP$^-$	0/48	1/48	a)	0/48	0/48	
BM huCD25$^+$/YFP$^-$	1/48	14/48	b)	0/48	0/48	
BM huCD25$^-$/YFP$^+$	0/48	0/48		0/48	3/48	e)
BM huCD25$^+$/YFP$^+$	0/48	0/48		0/48	0/48	
Thymus huCD25$^-$/YFP$^-$	0/48	3/48	c)	3/48	4/48	f)
Thymus huCD25$^+$	1/48	10/48	d)	0/48	3/48	g)
Thymus huCD25$^-$/YFP^{int}	0/48	0/48		0/48	0/48	
Thymus huCD25$^-$/YFP^{high}	0/48	0/48		23/48	48/48	h)

Table 6-3: BM and thymus of preTα-YFP/λ5-huCD25 double transgenic were sorted according to expression of YFP and huCD25 (Figure 6-31) and cultured cultivated as single or ten cells on OP9 and OP9-DL1 stromal cells. Wells containing colonies >100 cells were counted as positive. The phenotype was determined by FACS (Figure 6-34).

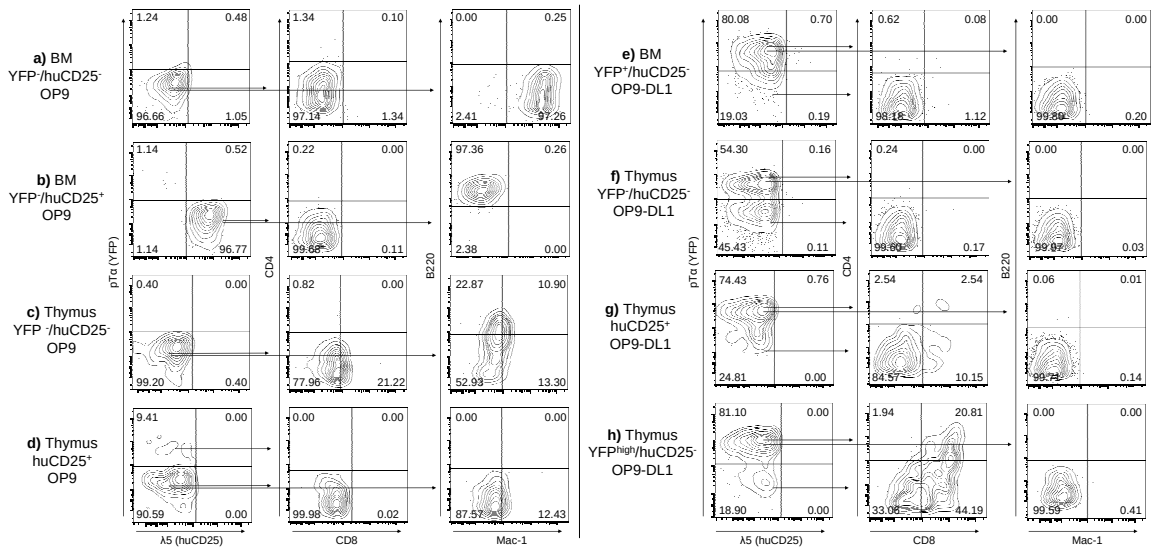

Figure 6-34: Expression of YFP and huCD25, and B-, T- and myeloid cell markers on cells that were grown from bone marrow (BM) and Thymus YFP/huCD25 populations sorted and cultivated for ten days as single or ten cells on OP9 or OP9-DL1 stromal cells.

6.4.8 Analysis of the in vivo developmental potential of YFP$^+$ and/or huCD25$^+$ cells

As *in vitro* analyses do not necessarily reflect the *in vivo* situation the reporter gene expressing populations from BM and thymus were also analysed for their potential to develop into B and/or T cells *in vivo* upon intravenous transplantation. The cells were FACS sorted twice and transplanted into sublethally irradiated immunodeficient Rag2$^{-/-}$ $\gamma_C^{-/-}$ mice. The mice were analysed 4 weeks after transplantation for the presence of B and T cells in the BM, spleen and thymus.

No B and T cells could be found in the mice deriving from the transplanted cells.

Probably, low number of cells transplanted into recipient mice as well as failed homing of the cells to the target organs are reasons for the failure.

7 Discussion

7.1 ES cells can develop into lymphoid, myeloid and erythroid cells *in vitro*

An *in vitro* culture system to develop different kinds of hematopoietic cell lineages, i.e. lymphoid, myeloid and erythroid cells from ES cells has been established. The culture can be subdivided into three periods. During the first phase, i.e. from day 0 to day 5, mesodermal-like cells expressing the mesodermal marker Flk-1 develop upon induction of differentiation from ES cells. During this first stage of development *in vitro* the OP9 stromal cells provide all cytokines and contacts so that no additional cytokines are needed. In the second period, i.e. from day 5 to day 10, the cells are subcultivated onto new stromal cell layers to induce the development of hematopoietic progenitor cells expressing the hematopoietic marker CD45 from the mesodermal cells. For that stage the cytokines SCF and Flt-3L are additionally needed. SCF alone is also sufficient for the appearance of $CD45^+$ hematopoietic cells but in that case the number of cells developing on day 10 is lower. However, it has been reported that Flt-3L induces the loss of long-term renewal capacity of HSCs and hematopoietic progenitors[203, 204]. Mutations causing constitutive expression of Flt-3 result in myeloproliferative disease whereas[205] Flt-3 as well as Flt-3L are dispensable for HSC maintenance in mouse bone marrow[206]. Therefore, it remains to be investigated in greater detail whether Flt-3L suppresses HSC formation in the differentiating ES cells.

From day 10 on dependent on the cytokines provided in the culture different hematopoietic lineages can be induced. This change of the culture conditions was critical. The culture conditions were first chosen to develop B cells which are known to allow long-term proB and preB cell proliferation in the presence of IL-7 and stromal cells [71-74]. This might be the most sensitive differentiation *in vitro*, since no or only very low numbers of B cells could be detected if the wrong basic media were provided. Whenever passaging the cells was done at the same time as changing the culture conditions no B cells developed, suggesting that these two changes might be too stressful for the cells. Therefore, after passaging the cells the same media was provided for 2 additonal days (from day 10 to day 12) before changing to the “B cell condition”. The emerging preB cells did not yield long-term proliferating preB cell lines in IL-7-containing media on OP9 stromal cells but differentiated upon activation with IL-4, IL-5 and anti-CD40 to become IgM^+ B cells. Hence, other stromal cell lines such as ST2 cells should be employed to see whether long-term proliferating preB cell lines can be established.

If the cells were cultured on OP9-DL1 cells they developed into DN as well as CD4/CD8 expressing T cells. Again, these DN cells, in contrast to DN cells developed from BM cells or isolated from the thymus, did not proliferate long-term in these cultures. This difference needs to be investigated in more detail. It is possible that *in vitro* ES cell-derived preB and preT cells are different because of the missing microenvironment provided *in vivo*.

Functional NK cells expressing perforin and granzyme B were developed upon addition of IL-2 and IL-15 on OP9 stromal cells.

Erythropoiesis was induced by addition of erythropoietin and SCF from day 10 on. The resulting erythrocytes expressed fetal as well as adult type globin, indicating that both primitive and definitive hematopoiesis had been induced in these cultures.

Osteoclasts, as one example of myeloid cells, were induced on ST2 cells, which provide M-CSF and RANKL in the presence of dexamethasone and 1α,25-dihydroxyvitamin D_3, and were detected by the expression of TRAP.

The *in vitro* differentiation of all hematopoietic cell lineages has been reproduced several times. This allowed developing a quantitative version of the assay of cellular development in order to monitor the differentiation of lymphoid, myeloid and erythroid cells with time, i.e. by determining the numbers of lymphoid, myeloid and erythroid cells that develop at various times after initiation of ES cell differentiation. The *in vitro* culture system should facilitate genetic studies of hematopoietic development, e.g. the identification of cellular and molecular mechanisms that may be defective in mutant ES cell lines.

7.2 iPS cells expressing elevated levels of Sox-2, Oct-4 and Klf-4 are severely reduced in their differentiation from mesodermal to hematopoietic progenitor cells

The established *in vitro* culture system was used to compare the efficiency of ES and iPS cells to develop into hematopoietic lineages.

iPS cells have been generated from hematopoietic progenitors present in bone marrow of 5-fluorouracil-treated mice, which proliferate *in vitro* in the presence of SCF and IL-6 [207, 208]. It was expected that iPS cells generated from hematopoietic progenitors might have epigenetic memory that might be close to the hematopoietic cells that were developed from the iPS cells *in vitro.* Such iPS cells might, therefore, be more efficient in the development of hematopoietic cell lineages *in vitro* and, upon transplantation *in vivo.*

The development of erythroid, myeloid as well as T-, B- and NK-lymphoid cells was severely reduced from iPS cells compared to ES cells. The most obvious differences were detectable for the development into the B cell lineage. While iPS A showed only 10 times lower efficiency, iPS clones B and C were 10000-fold less efficient and no preB cells were detectable from iPS D-G. By contrast the differences are not as severe for erythropoiesis and NK cell development. For most of the lineages iPS A proved to be the clone with the highest efficiency among the iPS cell clones. However, for osteoclastogenesis iPS B gave rise to as many mature cells as iPS A. These results indicate that different iPS clones might have different capacities to develop into the different hematopoietic cell lineages. However, all of them lower than ES cells.

As the initiation of development into all hematopoietic lineages was severly reduced from iPS cells, it seemed that a much lower number of common progenitors develop from those cells. The common stages, i.e. from day 0 to day 10 were therefore investigated in more detail.

Of the seven iPS cell clones four developed the same number, and two half the number of mesodermal Flk-1^+ cells seen with ES cells at day 5 of iPS cell differentiation. Thereafter, from day 5 onwards, all seven iPS cell lines generated much lower numbers of total and of $CD45^+$ hematopoietic lineage cells, when compared with the two ES cell lines. It appears that the severely reduced capacity of our differentiating iPS cell lines becomes manifest in the time between day 5 and 10 of *in vitro* development, i.e. during the development of hematopoietic progenitors from mesodermal progenitors.

It should be clear from our experimental results that our studies cannot clarify whether, and if so, how this defective development continues into the different hematopoietic cell lineages after day 10 of differentiation.

iPS cell generation and subsequent differentiation, either to whole mice or to differentiated cell lineages *in vitro*, may be the consequence of the loss or mutation of genes [40] or of epigenetic programming of the cells of origin, such as DNA methylation or miRNA expression, that is maintained in the iPS cells [35-41].

For the investigated iPS cell lines it appears more reasonable to expect that the strongly increased, irreversible expressions of Sox-2, Oct-4 and Klf-4 in the iPS cells and in the same, differentiating iPS cells at day 5 and 10 are major contributing factors that inhibit normal development from mesodermal-like cells at day 5 to hematopoietic progenitors at day 10. In this context it is worth noting that the iPS cell lines with the lowest increase in Sox-2 expression (lines A and B, Figure 6-16) have the best capacities to develop hematopoietic progenitors between day 5 and 10, and erythroid, myeloid and lymphoid cells thereafter. It is,

therefore, not clear whether all three transcription factors, or only one or two of them, suppress hematopoiesis. The analysis of the endogenous expression of Sox-2, Oct-4 and Klf-4 in all iPS cell lines in comparison to ES cells showed that they express similar levels. Therefore, I suggest that the hematopoietically suppressive actions are the direct result of the action of the transduced genes, and not an indirect activation of the endogenous loci by the transduced gene products.

7.3 Transplantable hematopoietic progenitors develop from mouse ES cells in a limited time window upon differentiation *in vitro*

As the *in vitro* system allows the differentiation of ES cells into all kinds of hematopoietic lineages, it was expected that an HSC or at least a hemtatopoietic progenitor cell stage exists in the culture. This was assayed by transplantation which should result in the repopulation of all hematopoietic lineages *in vivo.*

The *in vitro* system described in this thesis to differentiate transplantable hematopoietic progenitors from ES cells has been developed to provide a tool for successful short- and long-term engraftment of lymphoid and myeloid cells in immunodeficient mice.

This is in line with efforts that have been made to generate transplantable hematopoietic cells without genetic modifications of the ES cells [170-173]. This tissue culture conditions which were used here are close to those used by other laboratories in previous reports [138, 139]. They can be characterised and monitored by a series of structural and functional markers that appear and disappear at defined times after initiation of ES cell differentiation. Thus, Flk-1, CD41, CD45 are consecutively expressed and the cells can be induced to develop into lymphoid, erythroid and myeloid cells *in vitro*.

In this timed development a short time window between day 9 and 11 allows the appearance of transplantable hematopoietic progenitors.

Interestingly, the tissue culture conditions that were chosen and developed follow the programme in time of embryonic development so closely. During intra-embryonic development of the mouse the first hematopoietic progenitors are detected in the aorta-gonad-mesonephros region [42, 209-211] at embryonic day 7.5. First mesodermal cells appear at day 5, which is equivalent to day 8.5 of embryonic development. With the development of vascular endothelium at day 8.5 blood circulation is established so that from day 9.5, and with a peak at day 12, hematopoietic progenitor cells are detected in fetal liver, blood and placenta. [44]

Hematopoietic progenitors at day 9 in culture are equivalent to day 12.5 of embryonic development.

In contrast to development in the embryo development of hematopoietic progenitors in tissue culture occurs in a time-dependent fashion with a peak at culture day 10. At later time points, progenitor activity is lost. Therefore, the maintenance of these progenitors in the animal throughout life must depend on microenvironments in fetal liver and, later, in BM conditions that are not provided in the tissue culture conditions. While my experiments indicate sustained, long-term hematopoiesis from early progenitors in the transplanted recipients as one property of long-term reconstituting HSCs the long-term reconstitution in secondary transplantation could not be observed. Two possible explanations can be given for that failure. First, the numbers of secondarily transplanted ES-donor-derived BM cells could have simply been too low. Even with HSCs from normal BM it has been observed that secondary transplantations are less efficient [53]. Second, the ES-cell derived long-term reconstituting cells might not be true HSCs but another yet unidentified type of transplantable hematopoietic progenitor with long-term myeloid/lymphoid differentiation potential.

Consequences of the low efficiency of ES cell differentiation to hematopoietic progenitors and, especially lymphoid progenitors, are also evident in the inability of the normal ES-derived donor cells to populate the thymus. This is in contrast to T cell differentiation from ES cells *in vitro* differentiated for the same period of time, i.e. 10 days, that appear to occur in normal efficiencies. This inability to reconstitute the thymus, and consequently also T cells in peripheral lymphoid organs, is likely to result from the severe hypoplasia of the thymic stromal environment in $Rag2^{-/-}\gamma_C^{-/-}$ recipients that needs to cross-talk for its development with T-lymphoid progenitor cells entering the thymus from BM [212]. ES cells differentiated for 9, 10 or 11 days might have too few, or none of these progenitors to induce the development of thymic stroma. It is also possible that the hematopoietic progenitors generated from ES cells have a reduced potential to migrate and therefore cannot repopulate the thymus or that they cannot remain in their environmental niches in the thymus.

The B-lineage cell compartments in BM, spleen and peritoneum repopulated by ES cells differentiated *in vitro* for 9, 10 and 11 days were composed of comparable ratios of subpopulations of cells expected in these sites, though in lower cell numbers than in recipients receiving normal BM. This again suggests that the progenitor cells might be defective to fully reconstitute the B cell compartment, although their developmental potential appears fully developed when tested by *in vitro* differentiation. Again, migration from the injection site to the bone marrow, from the bone marrow niches to sites of further B cell development or the

stability of the B-lineage progenitors in these sites could reduce their overall B-cell generating potential.

In conclusion, the *in vitro* culture conditions that were used here to develop transplantable hematopoietic progenitors from ES cells can now be used in attempts to identify factors active in these progenitors or in their environment that could improve their homing to and stability in the BM and thymus, and in their long-term reconstituting potencies.

7.4 HOXB4 confers enhanced *in vivo* repopulation potential of ES cell-derived hematopoietic progenitor cells

HOXB4 has been shown to enable efficient generation of HSCs from ES cells [18-22, 163-167]. I have investigated whether HOXB4 transduction also enhances the *in vivo* repopulation potential of *in vitro* differentiated ES cells in the transplantation assays used in my thesis. The cells from day 5 to day 12 were transduced, selected and expanded in HSC-promoting culture conditions (containing IL-3, IL-6, SCF, TPO). While the cells can be transduced and expanded from all these days, the repopulation ability is restricted to cells from day 5 to day 10.

I found that HOXB4 transduction of differentiating ES cells enhances the repopulation efficiency of the cells. The percentage of successfully transplanted mice is similar to that of mice transplanted with non-HOXB4-modified cells. This indicated that cells with HSC-like qualities did not increase in numbers but were more efficient in hematopoietic differentiation especially to B cells. This might be due to a changed quality of the cells, e.g. stability of the cells *in vivo*, or due to a different composition of progenitor differentiation stages that are either, in the case of non-transduced cells, directly transplanted or, in the case of HOXB4 transduction, transplanted after selection. From those results it can be concluded that HOXB4 acts on progenitor cells that have endogenous repopulating ability (differentiated at day 9 to 11 of ES cell differentiation) as well as on earlier mesodermal progenitor cells (at day 5 to day 8). Cells beyond day 11 are still transducible by HOXB4 but do not yield multipotent hematopoietic reconstitution *in vivo* after transplantation.

Non-HOXB4-transduced ES cell differentiating between day 5 and 10 could be expanded in HSC-promoting culture conditions but were not able to repopulate immunodeficient mice (data not shown). This indicates that HOXB4 is keeping the stem cell characteristics of the cells. Stem cells, in this case HSCs, are characterised by their potential for both mutipotency and self-renewal [60, 213]. Differentiating HSCs give rise to MPPs which no longer possess self-

renewal ability yet keep full-differentiating potential [65, 214]. It is still impossible to maintain HSCs in culture by simply providing all factors by the culture condition, HSCs which may derive from ES cells even without HOXB4 transduction, directly continue their differentiation. The microenvironment provided by BM stromal cells under *in vivo* conditions cannot be provided sufficiently by OP9 stromal cells. HOXB4 appears to change the HSCs endogenously enabling their maintenance *in vitro,* independent of their proper niche. It appears to balance the capacity of the cells to self-renew and differentiate to the same extent, maybe by undergoing asymmetric cell divisions. HOXB4 is known to be a chromatin remodelling factor [215], but the detailed mode of action of HOXB4 is not known to date. HOXB4 overexpression in hematopoietic progenitors showed downregulation of genes that are preferentially expressed in mature hematopoietic cells [216] and revealed that HOXB4 acts by binding the DNA [215].

Another reason is that the *in vitro* differentiation was established and optimized based on the ability of the cells to proliferate in culture. On the other hand, most HSCs cycle very infrequently and primarily reside in the G_0 phase of the cell cycle under hematopoietic conditions [217]. Therefore, culture conditions which may favour the maintenance of HSCs should be focussed on keeping the cells quiescent, with limited proliferation that might be achieved by omitting Flt-3 ligand in culture which is known to be dispensable for HSC maintenance [206] and has here been shown to increase proliferation in the culture (see 6.1.1). In contrast, the action of HOXB4 appears to be artificial as it keeps the stem cell stage while inducing proliferation.

Qualitative differences were observed in mice transplanted with cells directly from the culture in comparison with those transplanted with HOXB4-transduced cells. In HOXB4 transplantations the thymus is repopulated with thymocytes, and the spleen contains normal ratios of T cells. This advantage might either be cell-intrinsic, i.e. the cells itself are more stable in their niches and can migrate more efficiently. It is also possible that a higher number of cells populate the thymus that are able to support each other, thus induce the development of the underdeveloped thymic microenvironment of $Rag2^{-/-}\gamma_C^{-/-}$ recipients more efficiently.

$CD19^+$ B cells in spleen and peritoneum contain a large number of IgM^- cells. In normal B cell development B cells without an IgM, i.e. a BCR on their surface, do not leave the BM but die. An abnormal development is also visible in myelopoiesis by the absence of $Gr1^+$ or $Mac1^+Gr1^+$ cells in spleen and peritoneum, in contrast to mice transplanted either with normal BM or with non-transduced ES-derived cells. It suggests deregulating actions of HOXB4. This HOXB4-mediated deviation needs to be investigated in detail in the future. Reversibly

inducible or conditionally deleting HOXB4 vectors should limit the action of HOXB4 to the phase of HSC and progenitor expansion *in vitro*.

The fact that cells can be transduced with HOXB4 and result in efficient repopulation of immunodeficient mice might be due to the expansion of the cells after transduction. Maybe transplantation on day 5 without further expansion results in tumour formation in recipients because on day 5 the mixture of cells contains many undifferentiated ES cells which are lost after subcultivation on day 5. The expansion process leads in the same way to loss of those undifferentiated cells and selects for HOXB4-responsive HSCs in the culture which are subsequently transplanted. As the mixture of differentiating cells do not include enough cells to be responsive to HOXB4, i.e. day 12 and thereafter, the frequency of repopulation decreases drastically.

Figure 7-1 summarises the repopulating abilty of wild-type, non HOXB4-transduced compared to HOXB4-transduced ES cell-derived cells.

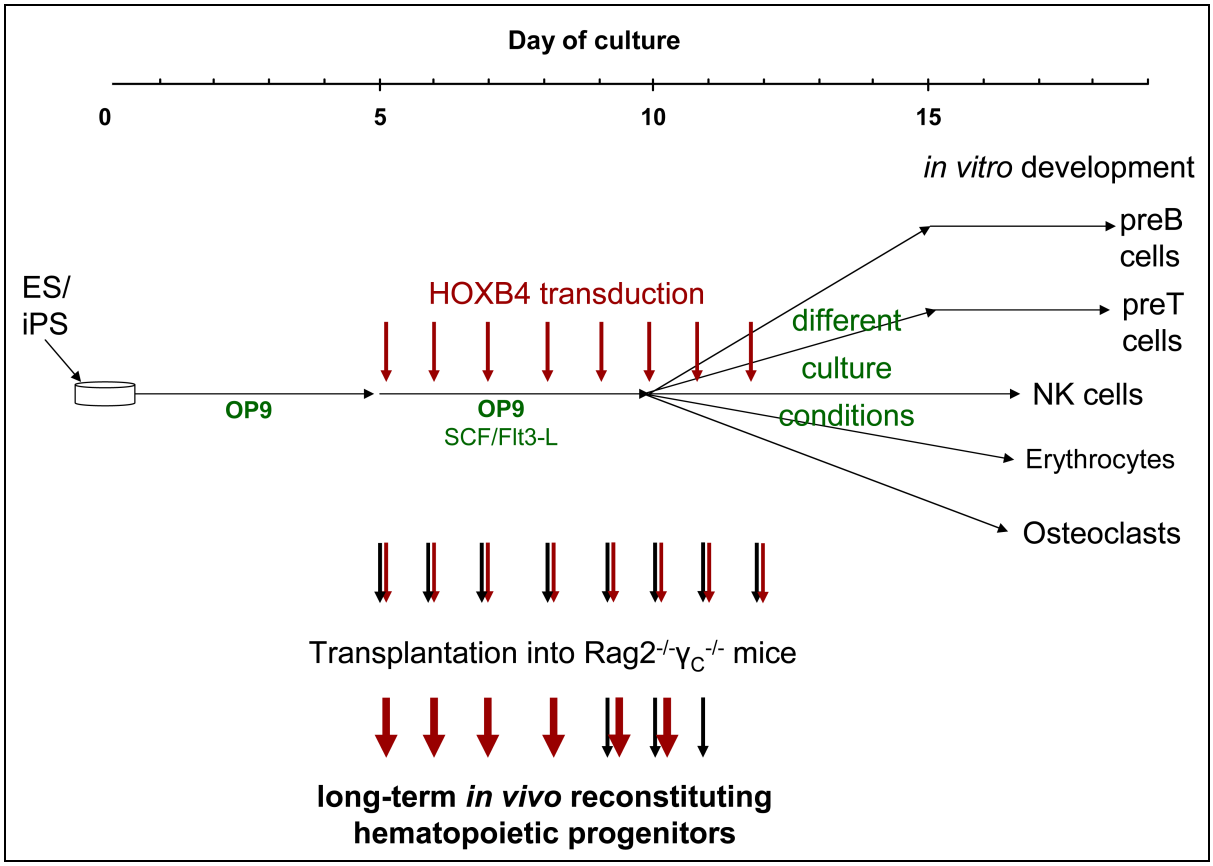

Figure 7-1: Reconstituting ability of wild-type and HOXB4-transduced ES cell-derived hematopoietic progenitors. Black horizontal arrows indicate timed transplantation of wild-type cells, red arrows show HOXB4 transduction with subsequent transplantation and reconstituion success. The size of the arrows correlate to the number of donor cells found in the transplanted hosts.

7.5 preTα-driven reporter gene expression marks T cell committed cells, while λ5-driven reporter gene expression marks B cell committed cells

BACs are large enough to contain several genes and are expected to maintain normal gene expression when inserted into the genome. In contrast to normal short transgenes BACs [218, 219] encode most, if not all, regulatory regions of a gene, as well as the *cis*-elements that define expression domains. Thus, BACs can be considered as complete expression units. Consequently, expression from BAC based vectors is less affected by the surrounding chromatin to their insertion site in a host genome. Using transgenesis technology, BACs have therefore been used as a surrogate to mouse gene-targeting (knock-in) technology, in which an allele is modified in stem cells before these are re-implanted into blastocysts.

The reporter BACs used in this study have been constructed so that no sequences were deleted. The fluorescent protein coding DNA was inserted at the start codon of the original gene. A polyA signal at the end of the inserted fluorescent protein gene avoided that fusion proteins were produced.

In previous studies expression of λ5 and preTα have been monitored in mice that express normal huCD25 transgenes, each separately [129, 130,134, 135]. In those studies the earliest progenitor in mice expressing transgenic huCD25 driven by the λ5 promotor is a $B220^{+}$ $CD19^{-}$ $CD27^{+}$ $ckit^{+}$ $Flt3^{+}$ $IL7R\alpha^{+}$ cell [129, 130]. preTα-driven huCD25-expressing cells in the BM have been found to have CLP potential, i.e. can develop into B and T lineage cells [134, 135]. RT-PCR analyses from CLPs, defined by surface markers, have shown that they coexpress preTα and λ5 in single cells [136]. The question remains whether this precursor can also be found using transgenes and whether the same cell is bipotent for B and T cell development.

Based on previous data I hypothesise that a subpopulation of CLPs exist that express both, preTα and λ5 in the same cell. This CLP can further differentiate into preB cells while switching off preTα and only expressing λ5. The only-λ5-expressing cell may still be flexible so that it may retain T cell developmental potential as it is in the case of $PAX5^{-/-}$ pro/preB I cells [220]. When CLPs leave the BM and enter the thymus λ5 expression is switched off while preTα-expression is kept on while further differentiating towards the T cell lineage. It is possible that CLPs already received signals to become T lineage cells within the thymus. Therefore, cells expressing preTα only may be found in the BM that are already T lineage committed. Cells are expected to downregulate preTα- and λ5-driven reporter gene expression as they further differentiate into mature B and T cells, respectively. The hypothesis is summarised in Figure 7-2. I aimed at testing this hypothesis as well as identifying the

phenotype of the reporter gene expressing cells by surface markers. Furthermore, the potential of the cells was tested in order to validate the hypothesis.

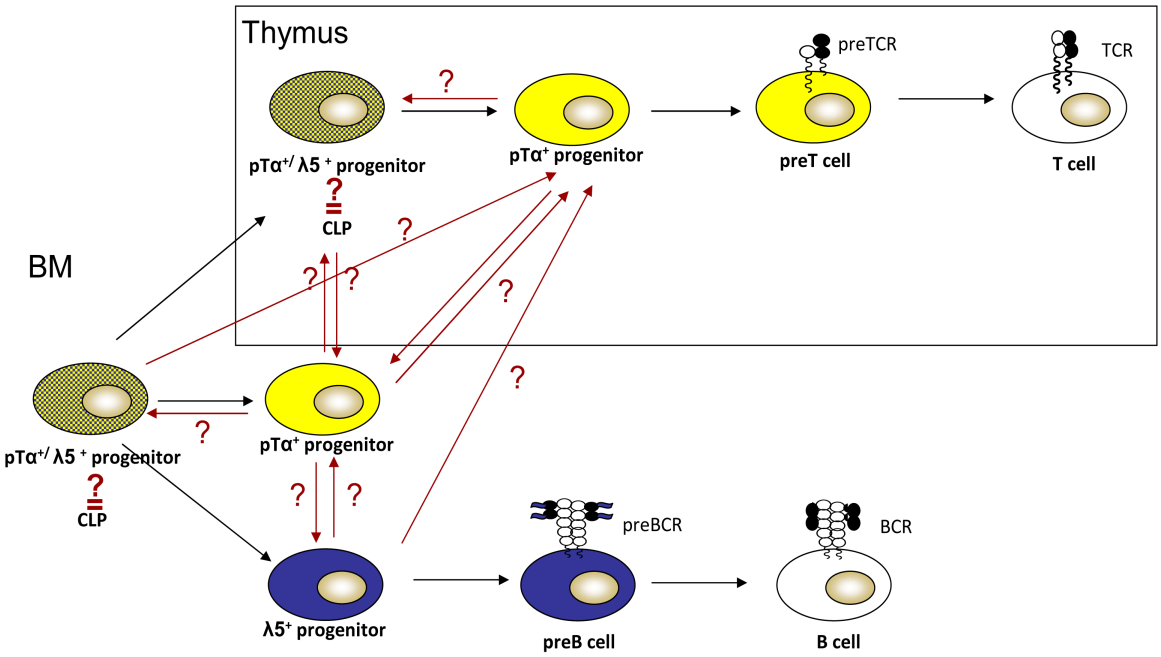

Figure 7-2: Hypothesis of preTα and λ5 driven reporter gene expression in B and T lineage cells and their progenitors. Blue - huCD25 expression, yellow - YFP expression.

In contrast to previous studies BAC clones allowing proper expression of reporter genes were used. To test the proper expression of preTα-driven YFP and λ5-driven GFP in the BACs they were tested first *in vitro* by generating BAC-transgenic ES cell lines that were differentiated into B and T cells *in vitro*.

BAC-transgenic mice were generated by pronuclear injection which is efficient enough to produce 10-40% transgenic littermates without drug selection and the need for germline-competent ES cells [221]. For the preTα-YFP BAC PCR-positive and functional littermates were generated. Injection of the λ5-GFP BAC resulted in PCR-positive mice but GFP^+ cells were never found. Classical transgenes hold the disadvantage to integrate randomly in the genome whereby, the integration site is critical as it plays a role whether the loci are closed or open and so the level of expression of the transgenes depends on that. In contrast, BACs are large enough to allow normal chromatin structure independent of gthe integration site. Therefore, the most likely explanation for the failure to generate functional λ5-GFP BAC-transgenic mice is that the BACs were injected without linearization. It might be that the BAC, in order to integrate, was broken within or very close to the reporter gene locus.

However, crossing the existing λ5-huCD25 (classical transgene) [129, 130] with the newly generated BAC-transgenic preTα-YFP mice resulted in double transgenic mice that were used for the following analyses.

Single- as well double-positive cells were found in the BM which is in line with previous studies that have preTα and λ5 expression by RT-PCR. The percentage of YFP^+ cells is similar to that found in the study with the preTα-huCD25 transgene [134, 135]. Additionally I could also show that some of the YFP^+ cells have a proB cell phentotype, some have a CLP phenotype. Previously it was found that some are $B220^+$, other are $ckit^+$, called CLP-1 and CLP-2 in the indicated study [134, 135]. I found exactly the same cells with the BAC transgene. Additonally I could show that some of the YFP^+ proB cells also express huCD25 (here as the reporter for λ5).

Based on that study I expected that at least some of the YFP^+ cells, single and or double positive for huCD25, found in the BM can develop into B cell as well as into T cells. However, the *in vitro* potential analysis showed YFP^+ $huCD25^+$ cells in the BM can only develop into T cells, though in very low frequency. However, they cannot develop into B cells *in vitro*. The double-positive cells identified in the BM can neither develop into B nor into T cells *in vitro*. $huCD25^+$ cells can only develop B cells but no T cells. This serves at the same time as an internal control of the assay. One reason for the failure of the expectation could be that the cells are stressed after being sorted twice. On the other hand it is possible that the *in vitro* system used here is not sufficient to generate B and T cells from cells with low potential. The same might be true for the cells from the thymus. The few $huCD25^+$ cells can only develop into B cells but not into T cells. YFP^+ cells can only develop into T cells, in which the YFP^{high} cells have the potential but not YFP^{int} cells. Here the reason is probably the different stages of development of the two YFP^+ populations. YFP^{int} cells are found within the DN1, DN2, DN4, DP and SP populations. The DN1 and DN2 populations are maybe not mature enough to grow under *in vitro* conditions only providing the DL-1 and IL-7 signal. In the same way the stages from the DN4 stage are too mature to grow under the indicated *in vitro* conditions. As those stages are not reported to express preTα I assume that downregulation of the reporter gene does not work properly in the transgenic mice or the half-life of the YFP protein is much higher than preTα. For the identification of a bipotent progenitor for B and T cell development this has no impact because only the onset of reporter gene expression is sufficient which seem to work properly.

Upon sorting double-negative cells do neither develop into B nor T cells. This result might be due to the low frequency within that population that contains many different types of cells,

very early progenitors as well as mature cells that cannot grow under *in vitro* provided preB cells conditions.

As *in vitro* systems do not provide the complete *in vivo* environment the potential analysis was also performed *in vivo*. The best method would be to directly inject the cells into the bone or thymus. It could be assumed that CLPs have the ability to move towards the BM and thymus. Therefore, intravenous injections might be sufficient. Injection of all populations did not show any results. This failure might be due to the very low numbers of injected cells or to the weakened quality of the cells after sorting. While *in vivo* assays are the gold standard for determining lineage potential of progenitor populations, they are limited by the cell numbers needed for reconstitution. *In vitro* assays on single cells must be perfomed to determine clonal lineage potential.

The differences found in my studies compared to other studies might be due to usage of BAC-based transgenes instead of normal short transgenes [134, 135]. Therefore, in further experiments the expression of the reporter genes has to be analysed and compared to the original gene expression by quantitative RT-PCR to test whether reporter gene expression parallels expression of the genes itself. Furthermore, in previous studies the T cell potential was not tested *in vitro* but by intrathymic injections which found bipotent preTα-driven huCD25^{+} cells in the BM [134, 135]. It might be that the progenitors are not able to home to the thymus but can develop into T cells inside the thymus providing the proper developmental microenvironment.

The B cell developmental potential was also investigated by *in vitro* cultivation in other studies, but as observed when establishing the culture conditions to differentiate ES cells *in vitro* it became clear that the culture system is very sensitive and therefore little differences might be critical for the differentiation potential.

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